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THE ROLE OF HISTIDINE-RICH GLYCOPROTEIN IN NECROTIC CELL CLEARANCE AND REGULATION OF DEGRADATIVE ENZYMES

IVAN KA HO POON

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY AT THE AUSTRALIAN NATIONAL UNIVERSITY

FEBRUARY 2009
Dedicated to my parents Poon Chi Kwong and
To Ling Fung, family, friends and mentors
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Declaration

All experimental work presented in this thesis has been performed and analyzed by the author unless otherwise stated. Dr. Craig Freeman assisted with the purification of plasma-derived HRG and the heparanase enzymatic activity assay. Dr. Robert Wood assisted with the binding of heparanase to cell surface receptors. Ms Anna Browne assisted with the stimulation of monocytes with LPS. All figures presented in this thesis have been designed and drawn by the author.

This thesis conforms to The Australian National University guidelines and regulations. The work presented in this thesis has not been submitted for the purpose of obtaining any other degree at this or other universities.

Ivan K. H. Poon  Prof. Christopher R. Parish  Dr. Mark D. Hulett
(Author) (Supervisor) (Supervisor)

Cancer and Vascular Biology Group
Division of Immunology and Genetics
The John Curtin School of Medical Research
The Australian National University

FEBRUARY 2009
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The Journey of my PhD - Thanks everyone!!
## Abbreviations

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<td>ABTS</td>
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<td>Apoptotic-cell-associated molecular patterns</td>
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<td>Australian National University</td>
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<td>APC</td>
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<td>BSA</td>
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<td>CIMPR</td>
<td>Cation-independent mannose-6-phosphate receptor</td>
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<td>CBA</td>
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<td>CLESH-1</td>
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<td>Centimeter</td>
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<td>CO₂</td>
<td>Carbon dioxide</td>
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<td>Cetylpyridium-chloride</td>
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<td>Dendritic cell</td>
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<td>Degrees Celsius</td>
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<td>DDW</td>
<td>Double distilled water</td>
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<td>Dulbecco’s modified Eagle’s medium</td>
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<td>DNA</td>
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<td>ds</td>
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<td>ECL</td>
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<td>Extracellular matrix</td>
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<td>EDTA</td>
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<td>eIF3</td>
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<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
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<td>FCS</td>
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<td>Fcγ receptor</td>
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<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<td>g</td>
<td>Gram</td>
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<td>gC1qR</td>
<td>Globular heads of complement C1q</td>
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<td>H-chain</td>
<td>Immunoglobulin heavy chain</td>
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<td>HERDS</td>
<td>Heterogenous ectopic ribonucleoprotein-derived structures</td>
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<td>HMDM</td>
<td>Human monocyte-derived macrophage</td>
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<td>HMGB1</td>
<td>High mobility group box 1</td>
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<td>HMWK</td>
<td>High-molecular-weight kininogen</td>
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<td>Histidine-rich glycoprotein</td>
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<td>Plasma-derived IgG-depleted HRG</td>
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<td>Horseradish peroxidase</td>
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<td>HRR</td>
<td>Histidine-rich region of HRG</td>
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<td>HS</td>
<td>Heparan sulfate</td>
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<td>Heat shock proteins</td>
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<td>Heparan sulfate proteoglycans</td>
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<td>[¹³H]-HS</td>
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<td>Intercellular adhesion molecule 3</td>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IgG&lt;sup&gt;HOG&lt;/sup&gt;</td>
<td>Co-purified IgG derived from HRG&lt;sup&gt;P&lt;/sup&gt;</td>
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<td>IL</td>
<td>Interleukin</td>
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<td>kDa</td>
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<td>Immunoglobulin light chain</td>
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<td>Lipopolysaccharide-binding protein</td>
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<td>LOX-1</td>
<td>Lectin-like oxidized LDL receptor-1</td>
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<td>LPA</td>
<td>Lysophosphatidic acid</td>
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<td>Membrane-attack complex</td>
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<td>Mannose-binding lectin</td>
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<td>Mean fluorescence intensity</td>
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<td>Major histocompatibility complex</td>
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<td>MMP</td>
<td>Matrix metalloproteases</td>
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<tr>
<td>MMR</td>
<td>Macrophage mannose receptor</td>
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<td>Macrophage scavenger receptor</td>
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<td>μg</td>
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<td>N-terminal region of HRG</td>
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<tr>
<td>ng</td>
<td>Nanogram</td>
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<td>N-hydroxysuccinimide</td>
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<td>Oxidized low-density lipoprotein</td>
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<td>Phosphatidic acid</td>
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<td>Phosphate buffered saline</td>
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<td>Human polymorphonuclear neutrophil</td>
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<td>Picomole</td>
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<td>Pattern recognition molecule</td>
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<td>Proline-rich region 1 of HRG</td>
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<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
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<td>PSN</td>
<td>Penicillin, streptomycin and neomycin</td>
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<td>PSR</td>
<td>Phosphatidylserine receptor</td>
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<td>Phosphatidylinositol</td>
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<td>RAGE</td>
<td>Receptor for advanced glycation end-products</td>
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<td>RF</td>
<td>Rheumatoid factor</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SAMPs</td>
<td>Self-associated molecular patterns</td>
</tr>
<tr>
<td>SAP</td>
<td>Serum amyloid protein</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SIP</td>
<td>Sphingosine-1-phosphate</td>
</tr>
<tr>
<td>SIPRα</td>
<td>Signal-regulatory protein α</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SMP</td>
<td>Skim milk powder</td>
</tr>
<tr>
<td>SPC</td>
<td>Sphingosylphosphorylcholine</td>
</tr>
<tr>
<td>SR-A</td>
<td>Scavenger receptor class A</td>
</tr>
<tr>
<td>SSC</td>
<td>Side scatter</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>t-PA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>t-PAR</td>
<td>Tissue plasminogen activator receptor</td>
</tr>
<tr>
<td>TSP</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td>u-PA</td>
<td>Urokinase plasminogen activator</td>
</tr>
<tr>
<td>u-PAR</td>
<td>Urokinase plasminogen activator receptor</td>
</tr>
<tr>
<td>V</td>
<td>Volts</td>
</tr>
<tr>
<td>W</td>
<td>Weight</td>
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<tr>
<td>Zn^{2+}</td>
<td>Zinc ion</td>
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</table>
Abstract

Histidine-rich glycoprotein (HRG) is an abundant multi-functional protein that is present in the plasma of many vertebrates. HRG has a multi-domain structure that allows the molecule to interact with many ligands including heparin, heparan sulfate (HS), Fcγ receptors (FcγR), plasminogen, fibrinogen, IgG, C1q, haem and Zn\(^{2+}\). The ability of HRG to interact with various ligands simultaneously has suggested that HRG can act as an adaptor molecule and regulate numerous biological processes such as immune complex/necrotic cell/pathogen clearance, cell adhesion, angiogenesis, coagulation and fibrinolysis. Although HRG is thought to play an important role in both immunity and vascular biology, the molecular mechanisms underpinning its action remain largely undefined. Thus, the aim of this thesis was to characterize the molecular components that are involved in HRG-mediated uptake of necrotic cells and to further examine the role of HRG in regulating degradative enzymes, such as plasminogen/plasmin and heparanase.

Studies outlined in Chapter 3 using a variety of techniques, including flow cytometry, ELISA and Western blotting, demonstrated that a complex consisting of both HRG and IgG is necessary to aid the phagocytosis of necrotic cells by the monocyte cell line THP-1. This process of necrotic cell uptake was found to be predominantly FcγRI- and HS-dependent. Intriguingly, results from direct binding experiments showed that HRG can potentially interact with both phospholipids and phosphorylated proteins exposed in necrotic cells. Furthermore, necrotic cell uptake enhanced by plasma-derived HRG also induced the release of pro-inflammatory cytokines such as IL-8 and TNF by THP-1 cells. Collectively, these data suggest that HRG has the unique property of complexing with IgG and initiating a pro-inflammatory innate immune response that promotes the clearance of necrotic cells at sites of tissue injury.

Chapter 4 describes experiments that investigated the proteolytic cleavage of human HRG by plasmin in detail and the effect of plasmin cleavage on various functions of HRG. SDS-PAGE and Western blotting studies revealed that HRG fragments generated...
by plasmin cleavage are held together by disulfide bonds and are not released from the HRG molecule under non-reducing conditions. Using flow cytometry- and ELISA-based binding studies, plasmin cleavage was found to reduce HRG binding to cell surface HS, but enhanced HRG binding to necrotic cells and to plasminogen immobilized on ELISA plates. However, both intact and plasmin-cleaved HRG enhanced plasminogen binding to heparin coated surfaces to a similar extent. Furthermore, the presence of heparin, Zn$^{2+}$ or acidic pH was demonstrated to protect HRG from plasmin cleavage. Based on these results, proteolytic cleavage of HRG by plasmin may provide a feedback mechanism to regulate the effects of HRG on the plasminogen/plasmin system, as well as other functions of HRG.

Lastly, Chapter 6 describes experiments designed to examine the ability of HRG to interact with human platelet-derived heparanase, an endo-β-glucuronidase that mediates the degradation of HS. ELISA studies demonstrated the direct binding of HRG to heparanase and showed that this interaction can be modulated by pH and the presence of hemin, heparin, HS, cardiolipin and the HS mimetic, PI-88. Using flow cytometry-based cell surface binding studies, HRG was also shown to modulate heparanase binding to cell surface receptors. Interestingly, heparanase enzymatic activity, at a range of pHs, was substantially enhanced by HRG. Based on these data, the direct interaction between HRG and heparanase can regulate the function of heparanase, potentially during both normal and pathological situations.

In summary, the studies presented in this thesis have delineated the molecular mechanisms underlying necrotic cell removal mediated by plasma-derived HRG and may have important implications for the development of autoimmune disease caused by defective clearance of dying/dead cells. In addition, the ability of HRG to regulate degradative enzymes, such as plasmin and heparanase, further supports the view that HRG may play an important role in angiogenesis, leukocyte migration and cancer metastasis.
Publications


Conference/meeting attended

Australasian Society for Immunology, 38th Annual Scientific Meeting, 2008, Canberra, Australia

Australian Society for Medical Research, Annual Research Conference, 2008, Canberra, Australia

Australasian Society for Immunology, 37th Annual Scientific Meeting, 2007, Sydney, Australia

12th Frank and Bobbie Fenner Conference, 2007, Canberra, Australia

13th International Congress of Immunology, 2007, Rio de Janeiro, Brazil

Research Center for Allergy and Immunology – The Japanese Society for Immunology, International Symposium, 2007, Pacifico Yokohama, Japan

2nd Research Center for Allergy and Immunology International Summer Program, 2007, Yokohama, Japan

Australian Society for Medical Research, Annual Research Conference, 2007, Canberra, Australia
Australasian Society for Immunology, 36th Annual Scientific Meeting, 2006, Auckland, New Zealand

Australian Society for Medical Research, Annual Research Conference, 2006, Canberra, Australia

Australasian Society for Immunology, 35th Annual Scientific Meeting, 2005, Melbourne, Australia

Australian Society for Medical Research, Annual Research Conference, 2005, Canberra, Australia

1. Oral presentation
2. Poster presentation
3. Chair for the Young Investigator’s Forum
The first part of this chapter provides a general introduction to the role of histidine-rich glycoprotein in immunity and vascular biology. The second part of this chapter reviews the molecular mechanisms of necrotic cell clearance in detail.
1.1 The role of histidine-rich glycoprotein in immunity and vascular biology: a brief update

1.1.1 Introduction

Histidine-rich glycoprotein (HRG), also known as histidine-proline-rich glycoprotein, is a ~75 kDa single polypeptide chain protein. HRG was first isolated and characterized from human serum in 1972 (Haupt and Heimburger, 1972; Heimburger et al., 1972), and later found to be present in the plasma of many vertebrates (Jones et al., 2005a) and in aquatic invertebrates (Nair and Robinson, 1999). Human HRG is synthesised in the liver (Koide et al., 1986) as well as in the brain (Ball-Rosen et al., 2007), and is present in plasma at the relatively high concentration of ~100-150 µg/ml (~1.5 µM). HRG has also been detected on the surface of leukocytes such as macrophages and monocytes (Sia et al., 1982), as well as in the α-granules of platelets and megakaryocytes (Leung et al., 1983).

The human HRG gene has been mapped to position 3q28-q29 on chromosome 3 (Koide, 1988; van den Berg et al., 1990), and is predicted to encode a 507 amino acid (Koide et al., 1986) multidomain protein consisting of two N-terminal regions with homology to cystatin-like domains (termed N1 and N2), a central histidine-rich region (HRR) flanked by two proline-rich regions (PRR1 and PRR2), and a C-terminal domain (C) (Fig. 1.1). Although HRG has been classified as a member of the cystatin supergene-family that are generally known as cysteine protease inhibitors (Koide and Odani, 1987), no protease inhibitory activity has been reported for HRG. As the name suggests, HRG contains an unusually high proportion of histidine and proline residues, each amino acid constituting approximately 13% of the total amino acids present in the protein. HRG also contains four intra-domain and two inter-domain disulfide bridges (Sorensen et al., 1993), and six predicted N-linked glycosylation sites (Haupt and Heimburger, 1972; Koide et al., 1986) (Fig. 1.1). The protein and gene structure of HRG has been recently reviewed in detail by Jones et al. (2005a).
A variety of molecules have been shown to interact with HRG, including haem (Katagiri et al., 1987), Zn$^{2+}$ (Morgan, 1981), plasminogen (Lijnen et al., 1980), fibrinogen (Leung, 1986), thrombospondin (TSP) (Walz et al., 1987), IgG (Gorgani et al., 1997), C1q (Gorgani et al., 1997) and heparin (Heimburger et al., 1972). HRG can also interact with various cell-associated molecules including Fcγ receptors (FcγR) (Gorgani et al., 1999b), cell surface heparan sulfate (HS) (Jones et al., 2004b), tropomysin (Guan et al., 1997).
an undefined T-cell receptor (Saigo et al., 1989), DNA (Gorgani et al., 2002) and cytoplasmic ligand(s) exposed on necrotic cells (Jones et al., 2005b). Schematic representation of HRG domains and the predicted ligand binding sites are summarized in Figure 1.1. Furthermore, based on the modular architecture of HRG, it has been proposed that HRG may act as a bridging or adaptor molecule that interacts with multiple ligands simultaneously through several independent binding sites (Borza et al., 1996). Collectively, HRG can potentially regulate numerous biological processes, with the first part of this chapter focusing on the physiological role of HRG in immunity and vascular biology (summarized in Fig. 1.2).

1.1.2 The role of HRG in immunity

1.1.2.1 HRG modulates the formation of immune complexes

Formation of immune complexes (IC) plays an important role in a normal immune response, whereby specific antibodies form complexes with target antigen and facilitate the clearance and neutralization of microorganisms or foreign substances (Schifferli and Taylor, 1989). However, if IC are not cleared properly from the circulation, their deposition at target tissues could result in pathological diseases such as arthritis, vasculitis and glomerulonephritis (Schifferli and Taylor, 1989). Besides components of the complement system such as C1q, HRG is another plasma protein that has been shown to interact with IgG and IC, subsequently regulating the clearance and formation of insoluble IC (IIC) (Gorgani et al., 1997; Gorgani et al., 1999b; Gorgani et al., 1999a; Gorgani et al., 1999c).

Figure 1.2. The role of HRG in immunity and vascular biology. HRG has been implicated in numerous biological processes through its ability to interact with various ligands. The proposed physiological roles of HRG can be subdivided into different categories as indicated in the figure. DNA, deoxyribonucleic acid; ECM, extracellular matrix; FcγR, Fcγ receptors; HRR, histidine-rich region; HS, heparan sulfate; IIC, insoluble immune complexes; LPS, lipopolysaccharide; RF, rheumatoid factor; TSP, thrombospondin.
Human HRG was first found to interact with both rabbit and human IgG (Gorgani et al., 1997), and later shown to bind different IgG subclasses and IgG molecules containing κ and λ light chains with different affinities (Gorgani et al., 1999c). HRG binding to IgG is dependent on its N-terminal domain and possibly involves HRG interacting with the F(ab) region of IgG (Gorgani et al., 1997). Most significantly, the HRG-IgG interaction seems to play an important role in regulating IIC formation and clearance. Studies by Gorgani et al. (1997) demonstrated that HRG is the key component of human plasma that is able to inhibit the formation of IIC. Furthermore, HRG can also prevent the formation of IIC generated by rheumatoid factor (an auto-anti-Fc antibody) and human IgG, possibly by masking the epitopes on IgG recognized by rheumatoid factor (RF) (Gorgani et al., 1999a). HRG can also aid the solubilization of already formed IIC (Gorgani et al., 1999a), as well as modulating the binding of IC to monocytes (Gorgani et al., 1999b).

1.1.2.2 Regulation of FcγR function by HRG

Leukocyte Fc receptors play a vital role in linking the humoral and cellular arms of the immune system by facilitating the interaction of antigen-specific antibodies with non-specific effector cells that express various Fc receptors. In humans, there are three classes of IgG binding FcγR, namely FcγRI, FcγRII and FcγRIII. These FcγR are expressed at differential levels on a variety of cell types (e.g. monocyte, macrophage, granulocyte, NK cell, platelet, dendritic cell and B cell) and regulate diverse biological processes (Ravetch and Bolland, 2001). Interestingly, HRG has been shown to regulate the expression and functions of FcγR (Chang et al., 1992; Chang et al., 1994; Gorgani et al., 1999b).

Initially, HRG was demonstrated to modulate FcγR (mainly FcγRII) mediated phagocytosis of IgG-opsonized sheep erythrocyte in a biphasic manner depending on the duration of pre-treatment of macrophages with HRG (Chang et al., 1992). Later studies by Chang et al. (1994) suggested that the effects of HRG on FcγR-dependent phagocytosis is attributed to the regulation of FcγRII expression and protein synthesis by
HRG. Furthermore, HRG was found to regulate monomeric IgG binding to FcγRI through direct interaction with FcγRI (Gorgani et al., 1999b). Although the *in vivo* role of HRG in modulating FcγR function is unclear, studies have demonstrated the importance of HRG in facilitating the clearance of IC (Gorgani et al., 1999b) and apoptotic cells (Gorgani et al., 2002) via an FcγR-dependent mechanism.

**1.1.2.3 HRG binds and facilitates the removal of apoptotic and necrotic cells**

Under normal physiological conditions, rapid removal of dying/dead cells (e.g. apoptotic and necrotic cells) from the circulation and tissues by phagocytic cells plays a critical role in maintaining tissue homeostasis and turnover. Impaired dying/dead cell clearance could result in the exposure of antigenic intracellular molecules, which can lead to the development of autoimmune diseases such as systemic lupus erythematosus (SLE) (Michlewska et al., 2007). A vast number of cell surface receptors and plasma opsonins have been implicated in the clearance of apoptotic and/or necrotic cells (reviewed in detail in Section 1.2 below). Recently, plasma-derived HRG was shown to bind and aid the uptake of apoptotic cells (Gorgani et al., 2002), as well as necrotic cells (Jones et al., 2005b).

Studies by Gorgani et al. (2002) showed that HRG binds strongly to late apoptotic cells compared to viable or early apoptotic cells, possibly by recognizing naked DNA exposed during apoptosis. Consistent with previous observations by Gorgani et al. (1999b), HRG binds FcγRI on human monocyte-derived macrophages (HMDM) and subsequently functions as a bridging molecule to augment the uptake of apoptotic cells via a FcγRI-dependent mechanism (Gorgani et al., 2002). The authors also demonstrated that, like components of the complement system (Mevorach et al., 1998), HRG is a key factor in normal human serum-dependent phagocytosis of apoptotic cells (Gorgani et al., 2002). Similarly, studies by Jones et al. (2005b) demonstrated that, in addition to cell surface HS, HRG binds strongly to cytoplasmic ligand(s) exposed on permeabilized necrotic cells via its N1N2 domain. However, HRG-mediated necrotic cell uptake is independent of cell surface HS, FcγRI and FcγRIIA on phagocytes (Jones et al., 2005b).
Interestingly, HRG has been reported to interact with Clq (Gorgani et al., 1997) and TSP (Leung et al., 1984; Silverstein et al., 1985; Walz et al., 1987), both of which are also involved in the uptake of apoptotic and necrotic cells (Krysko et al., 2006). Therefore, these opsonins may work in concert to potentiate the removal of dying/dead cells and determine the subsequent immune response.

1.1.2.4 Antimicrobial and endotoxin-neutralizing properties of HRG

The innate immune system often utilizes pattern recognition molecules (PRMs) (e.g. CD14, C-reactive protein (CRP), Clq and mannose binding lectin (MBL)) that recognize conserved molecular patterns on pathogens or dying/dead cells and aid their clearance by phagocytes (Gregory and Devitt, 2004). Besides the role of HRG in detecting and removing dying/dead cells (as described in Section 1.1.2.3 above), HRG and peptides derived from the HRR of HRG have been shown to bind to and exert antimicrobial effects against both bacteria (Rydengard et al., 2007) and fungi (Kacprzyk et al., 2007; Rydengard et al., 2008).

Similar to other histidine-rich (Bulet et al., 2004) and heparin-binding peptides (Andersson et al., 2004), peptides derived from the HRR of HRG, such as the 20mer peptide (GHHPH)$_4$ were able to bind to heparin (Rydengard et al., 2007) and exert antimicrobial activity against the Gram-positive bacteria Enterococcus faecalis, the Gram-negative bacteria Escherichia coli (Rydengard et al., 2007) and the fungus Candida albicans (Kacprzyk et al., 2007; Rydengard et al., 2008) under acidic conditions or in the presence of Zn$^{2+}$. Furthermore, unlike the closely related cystatin superfamily member high-molecular-weight kininogen (HMWK), which requires proteolytic cleavage by elastase to generate antimicrobial fragments/peptides (Nordahl et al., 2005), intact plasma-derived and recombinant HRG were able to exert antibacterial (Rydengard et al., 2007) and antifungal effects (Rydengard et al., 2008). In the presence of Zn$^{2+}$ or at low pH, intact HRG was able to bind and trigger membrane destabilization, as well as the release of the cytoplasmic contents of whole bacteria and fungi. However, the HRR seems to be essential for this process as recombinant HRG containing only the
two N-terminal cystatin-like regions showed no antimicrobial activity (Rydengard et al., 2007; Rydengard et al., 2008). Recently, the antifungal role of HRG was validated in vivo using HRG deficient (HRG<sup>-/-</sup>) mice, in which HRG<sup>-/-</sup> mice were more susceptible to *Candida albicans* infection than HRG<sup>++/+</sup> C57BL/6 mice (Rydengard et al., 2008).

Cationicity of many antimicrobial peptides are known to be critical for the initial electrostatic attraction of peptides to the highly electronegative surface of bacteria and fungi, as well as the ability of peptides to traverse the microbial plasma membrane to aid pathogen killing via either membrane or non-membrane disruptive mechanisms (Yount et al., 2006). While no bacterial ligand(s) of HRG have yet been identified, one could speculate that the cationicity of the HRR may play an important role since the antimicrobial effects of HRG and the HRR-derived peptides were highly dependent on low pH or the presence of Zn<sup>2+</sup> (Rydengard et al., 2007; Rydengard et al., 2008), both of which impose a net positive charge on the molecule. Interestingly, a synthetic peptide containing the histidine-rich consensus sequence of HRG was able to neutralize the lipopolysaccharide (LPS)-induced pro-inflammatory response by dampening LPS-induced IL-8 production by CD14-transfected THP-1 cells (Bosshart and Heinzelmann, 2003). Despite the lack of direct evidence of an interaction between HRG and the anionic bacterial component LPS, HRG may act as a PRM that recognizes anionic molecules exposed on microorganisms and facilitate their removal either via direct killing or phagocytosis.

1.1.2.5 **HRG modulates cell adhesion**

Immune cells use a variety of cell adhesion molecules to mediate communication with other cells (e.g. interaction between T cells and antigen presenting cells or target cells) (Lebedeva et al., 2005), as well as migration to inflammatory sites (e.g. adhesion and transmigration through the endothelium during an immune response) (Ley et al., 2007). Previous studies have suggested that HRG may play either a positive or a negative role in regulating cell adhesion of immune cells such as murine T cells and macrophages (Sia et al., 1982; Chang et al., 1992; Lamb-Wharton and Morgan, 1993; Olsen et al., 1996).
In vitro studies with a T cell line showed that the presence of HRG can enhance the adhesion and spreading of activated T cells on plastic tissue culture plates (Lamb-Wharton and Morgan, 1993). The combination of HRG and Zn\(^{2+}\) can also promote homotypic adhesion between T cells in culture (Olsen et al., 1996). Therefore, HRG may positively regulate cell adhesion. In contrast, other studies have observed an inhibitory effect of HRG on the adhesion of immune cells. HRG was shown to inhibit the formation of autorosettes (cell adhesion between murine lymphocytes and autologous erythrocytes) (Rylatt et al., 1981; Sia et al., 1982). Furthermore, studies by Olsen et al. (1996) showed that in the presence of Zn\(^{2+}\), HRG inhibits the adherence of a T cell line to tissue culture plastic, as well as extracellular matrix (ECM) components (e.g. laminin, collagen, or fibronectin) coated to culture dishes. Similarly, Chang et al. (1992) found that prolonged treatment of macrophages with HRG reduces their spreading and adherence to plastic wells. Although the molecular mechanisms underlying the diverse effects of HRG on cell adhesion are unclear, recent studies have demonstrated that HRG and peptides derived from the HRR can modulate signal transduction events that are important in regulating cytoskeletal organization of endothelial cells (Dixelius et al., 2006; Lee et al., 2006), as well as interfering with \(\alpha\beta_3\) integrin-mediated adhesion of endothelial cells to vitronectin (Dixelius et al., 2006).

1.1.3 The role of HRG in vascular biology

1.1.3.1 Anti-angiogenic and pro-angiogenic properties of HRG

Angiogenesis, the formation of new blood vessels from pre-existing vasculature, is essential in maintaining the delivery of sufficient oxygen and nutrients to all cells within an organism (Carmeliet, 2003). Therefore, angiogenesis is vital in physiological processes such as embryogenesis and wound healing, and in a variety of malignant and immune disorders (Carmeliet and Jain, 2000; Carmeliet, 2003). Angiogenesis is tightly regulated by a multitude of endogenous activators and inhibitors, and involves a variety of cell types (Carmeliet and Jain, 2000; Carmeliet, 2003). HRG and peptides derived from the HRR of HRG have been shown to exhibit both pro- and/or anti-angiogenic properties, depending on the
experimental systems used (Simantov et al., 2001; Juarez et al., 2002; Donate et al., 2004; Guan et al., 2004; Simantov et al., 2005; Dixelius et al., 2006; Lee et al., 2006).

One potential molecular mechanism underpinning the pro-angiogenic effects of HRG involves its strong interaction with TSP. TSP is a multifunctional homotrimeric α-granule glycoprotein that inhibits angiogenesis through its interaction with the signalling receptor CD36, which delivers an anti-angiogenic signal that blocks basic fibroblast growth factor (bFGF)-induced angiogenesis (Simantov et al., 2001). HRG was initially found to bind TSP with high affinity (Leung et al., 1984) and was able to form a trimolecular complex with plasminogen and TSP (Silverstein et al., 1985). Recently, studies by Simantov et al. (2001) showed that HRG contains two CLESH-1 motifs that resemble the TSP binding motif on CD36, and HRG was proposed to aid bFGF-induced angiogenesis by interfering with TSP-CD36-mediated anti-angiogenic signalling (Simantov et al., 2001; Simantov et al., 2005).

In contrast, HRG and peptides derived from the HRR of HRG (notably the 20mer peptide (GHHPH)₄) were able to induce potent anti-angiogenic effects both in vitro and in vivo via a number of mechanisms. HRG could inhibit angiogenesis by modulating various signalling events that are important for endothelial cell survival, proliferation and cell migration. For example, HRG was shown to inhibit proliferation and induce apoptosis in endothelial cells in vitro via the activation of caspase-3 (Juarez et al., 2002). Although studies by Olsson et al. (2004) also showed that tumours in HRG-treated mice have reduced vascularization, increased apoptosis and decreased proliferation, HRG had no apparent effects on the survival or growth of endothelial cells in vitro. This was possibly due to an unlimited supply of oxygen and nutrients in in vitro cultures compared to the vasculature of tumor cells in vivo. Furthermore, HRG can directly transduce anti-angiogenic signals though the interaction with tropomyosin expressed on endothelial cells following bFGF activation (Guan et al., 2004). HRG can also reduce chemotaxis of primary endothelial cells (Olsson et al., 2004), possibly caused by the rearrangement of focal adhesion (Olsson et al., 2004; Dixelius et al., 2006; Lee et al., 2006), disruption of cytoskeletal organization (Dixelius et al., 2006; Lee et al., 2006),
inhibition of tube formation (Lee et al., 2006) and reduction in cell attachment (Olsson et al., 2004; Dixelius et al., 2006). The molecular mechanism underlying the effects of HRG on the endothelial cell cytoskeleton was suggested to involve signalling events mediated through integrin-linked kinase (Dixelius et al., 2006) and focal adhesion kinase (Olsson et al., 2004; Dixelius et al., 2006; Lee et al., 2006), which could modulate processes that are important for angiogenesis such as lamellipodia formation, polarization and migration (Dixelius et al., 2006). Importantly, the anti-angiogenic properties of HRG can be mediated solely via the HRR/PRR region (Juarez et al., 2002) or peptides derived from the HRR of HRG (Donate et al., 2004; Olsson et al., 2004; Dixelius et al., 2006; Lee et al., 2006).

The angiogenic role of HRG is further complicated by its ability to regulate degradative enzymes such as the plasminogen/plasmin system that could in turn modulate the formation of new vessels during angiogenesis. Extracellular proteases such as matrix metalloproteases (MMP) and the plasminogen/plasmin system are critical in the remodelling of the ECM during vessel sprouting and the resolution phase of angiogenesis (Pepper, 2001). Numerous investigators (Lijnen et al., 1980; Saez et al., 1995; Borza and Morgan, 1997; Borza et al., 2004; Jones et al., 2004a) have demonstrated a strong interaction between HRG and plasminogen, which can either positively or negatively regulate the activation of plasminogen to the serine protease plasmin via plasminogen activators (see also Section 1.1.3.2 below). Furthermore, HRG can also regulate the function of another ECM remodelling enzyme heparanase, an endo-β-D-glucuronidase, by masking the heparanase cleavage sites of ECM HS (Freeman and Parish, 1997).

In addition to the above mechanisms, HRG could also exhibit pro- or anti-angiogenic effects through controlling the availability of HS-binding growth factors such as FGF (an angiogenesis activator) to endothelial cells. Studies by Brown and Parish (1994) have demonstrated that HRG can compete with FGF for binding HS on cell surfaces, which inhibits the mitogenic activity of FGF by preventing cell surface HS serving as a co-receptor for FGF. Alternatively, HRG could aid angiogenesis by displacing
biologically active FGF sequestered by HS component of the ECM (Brown and Parish, 1994). As described earlier, the ability of HRG to protect the heparanase-sensitive areas of HS also suggests a potential role for HRG in either limiting angiogenesis by inhibiting heparanase-mediated release of HS-binding growth factors from the ECM HS, or facilitating angiogenesis by preventing cleavage of endothelial cell surface HS which serves as a co-receptor for FGF signalling (Freeman and Parish, 1997).

Collectively, HRG has been proposed to modulate angiogenesis via a diverse array of mechanisms. Further in vivo studies using HRG-/- mice are necessary to dissect whether endogenous HRG functions predominately as an activator or inhibitor of angiogenesis.

1.1.3.2 Regulation of coagulation and fibrinolysis via HRG

Blood clotting (coagulation) and the dissolution of fibrin clots (fibrinolysis) are tightly regulated by a range of substrates, activators, inhibitors, cofactors, and receptors to ensure precise prevention of blood loss and unnecessary blockage of vessels (Cesarman-Maus and Hajjar, 2005). During coagulation, exposure of damaged vascular surfaces results in the initial formation of a ‘platelet plug’, which ultimately leads to the generation of thrombin and the formation of a thrombus through thrombin-mediated conversion of fibrinogen to fibrin, and by platelet activation (Triplett, 2000). During fibrinolysis, the key fibrinolytic protease plasmin, is generated via proteolytic processing of the plasma zymogen plasminogen by tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) (Cesarman-Maus and Hajjar, 2005). Interestingly, HRG has been shown to interact with components of both the coagulation and fibrinolytic systems, thus potentially playing an important role in regulating hemostasis (Heimburger et al., 1972; Lijnen et al., 1980; Lijnen et al., 1983a; Leung, 1986; Borza and Morgan, 1997).

Second to anti-thrombin III, HRG is one of the most abundant heparin-binding proteins in human plasma. HRG binds heparin with high affinity (Heimburger et al., 1972; Lijnen et al., 1983a) and was shown to neutralize the anti-coagulant activity of heparin
by preventing the formation of heparin-anti-thrombin III complexes that inhibit activated coagulation factors such as thrombin (Lijnen et al., 1983b; Lijnen et al., 1983a; Lijnen et al., 1983c; Lijnen et al., 1984). Furthermore, the presence of Zn\(^{2+}\) that is released by activated platelets (Aktulga, 1974), can potentiate the interaction between HRG and heparin (Lijnen et al., 1983a; Jones et al., 2004b), and the ability of HRG to neutralize heparin (Kluszynski et al., 1997; Fu and Horn, 2002). Interestingly, HRG can also interact with fibrinogen and be incorporated into fibrin clots (Leung, 1986). Although the authors observed that HRG had no effect on the extent of fibrinogen conversion into fibrin by thrombin during the formation of fibrin clots, the presence of HRG did retard the rate of conversion of fibrinogen to fibrin (Leung, 1986).

As mentioned earlier (see Section 1.1.3.1), HRG binds strongly to the lysine-binding site on plasminogen (Lijnen et al., 1980), possibly via its C-terminal lysine residues (Saez et al., 1995; Borza and Morgan, 1997). Initially, HRG was proposed to be an anti-fibrinolytic agent by interfering with plasminogen interacting with binding partners that are important for its activation (Lijnen et al., 1980), such as fibrinogen, fibrin, integrin \(\alpha_m\beta_2\) and annexin 2 (Cesarman-Maus and Hajjar, 2005). Controversially, HRG can either inhibit fibrinogen-dependent plasminogen activation (Borza et al., 2004) or has no apparent effect on fibrin-dependent plasminogen activation (Horne et al., 2000) in solution. In contrast, HRG has also been suggested to function as a soluble plasminogen receptor that aids plasminogen activation by tethering plasminogen to glycosaminoglycan (GAG) coated surfaces (Borza et al., 2004) and cell surfaces (Jones et al., 2004a).

The physiological role of HRG in hemostasis was to some extent resolved by the generation of HRG\(^{-}/-\) mice by Tsuchida-Straeten et al. (2005), this study suggesting that HRG has both anti-coagulation and anti-fibrinolytic properties \textit{in vivo}. Notably, HRG\(^{-}/-\) mice showed a significantly shorter plasma prothrombin time as well as shorter bleeding times than HRG\(^{+/+}\) mice, which indicates accelerated extrinsic clotting in HRG\(^{-}/-\) mice (Tsuchida-Straeten et al., 2005). HRG\(^{-}/-\) mice also have enhanced fibrinolysis, whereby
fibrin clots are lysed more rapidly in HRG<sup>−/−</sup> mice in comparison with HRG<sup>+/+</sup> mice (Tsuchida-Straeten <i>et al.</i>, 2005).

1.1.3.3 <i>The role of HRG in cancer progression</i>

Tumorigenesis is known to be a multistep process that involves a variety of genetic alterations, which can progressively transform normal cells into aggressive malignant derivatives (Hanahan and Weinberg, 2000). During the process of transformation, cancer cells often hijack normal physiological processes to facilitate growth and metastasis, such as the ability to induce angiogenesis to maintain tumour survival (Carmeliet and Jain, 2000) and to invade adjacent or distal tissues by modulating cell adhesion molecules (Okegawa <i>et al.</i>, 2004) and extracellular degradative enzymes (Pepper, 2001). Since previous studies summarized above have suggested that HRG can participate in the regulation of angiogenesis, cell adhesion, cell proliferation and the remodelling of the ECM, it is of great interest to ascertain whether endogenous HRG plays a role in cancer progression.

1.1.4 <i>Concluding remarks</i>

In healthy human adults, soluble proteins of the innate immune system (e.g. C1q), and proteins that maintain the homeostasis of the vascular system (e.g. plasminogen, fibrinogen and anti-thrombin III) are often present at a relatively high concentration (>100 µg/ml) in the plasma to provide immediate responses to foreign pathogens and vascular damage. Likewise, HRG is present in human plasma at approximately 100-150 µg/ml and has been implicated in immunity and vascular biology as described in the first part of this chapter. Interestingly, HRG was found to be a negative acute phase reactant (Saigo <i>et al.</i>, 1990a), and circulating HRG levels are significantly lower during acute inflammation (Saigo <i>et al.</i>, 1990b) and in patients with SLE (Castel, 1988). These observations suggest that HRG is actively involved in acute inflammation as well as chronic autoimmune diseases. Although a number of clinical studies have reported that elevated or reduced HRG levels in patients are also associated with various thrombotic
diseases (Angles-Cano et al., 1993; Castaman et al., 1993; Hoffmann et al., 1993; Hennis et al., 1995; Souto et al., 1996; Shigekiyō et al., 1998), families with congenital HRG deficiencies (20% to 35% of normal plasma level) fail to show any apparent abnormalities in routine laboratory assays of hemostatic and immunologic function, suggesting that HRG at 20% normal plasma levels is adequate to maintain normal physiological functions (Shigekiyō et al., 1995). Alternatively, it is also possible that HRG only plays a minor regulatory role in various biological processes or that its functions are redundant.

In summary, multiple lines of evidence have indicated that HRG is an important plasma protein for immunity and vascular biology. Future studies using HRG<sup>+/−</sup> mice in various <i>in vivo</i> models are essential to validate the current observations. For example, it will be of considerable interest to see whether HRG<sup>+/−</sup> mice are more susceptible to bacterial infections and autoimmune diseases due to a deficiency in pathogen and dying/dead cell clearance. Similarly, HRG<sup>+/−</sup> mice may be more or less prone to cancer development caused by a dysregulation of degradative enzymes and/or angiogenesis. Such studies are in their infancy but the recent generation of an HRG<sup>+/−</sup> mouse (Tsuchida-Straeten et al., 2005) should greatly aid these investigations.

1.2 Molecular mechanisms of dying/dead cell clearance

As described in Section 1.1 above, HRG plays an important role in handling the removal of late apoptotic and necrotic cells (Section 1.1.2.3), as well as bacteria and fungi (Section 1.1.2.4). Thus, the molecular mechanisms underpinning necrotic cell uptake, the striking similarities between pathogen and dying/dead cell removal, and the effects of phagocytosis on the subsequent immune response will be discussed in detail below.

1.2.1 Introduction

The immune system is constantly under pressure to accurately detect and distinguish foreign materials/pathogens (non-self) from normal healthy tissues (self), and making an
appropriate immune response to non-self molecules via a range of effector mechanisms. It is equally important for the immune system to distinguish healthy viable cells (self) from dying/dead cells (altered-self) during the course of tissue remodelling or tissue injury to prevent the release of intracellular molecules that may damage neighbouring cells and/or stimulate an immunogenic response against self (i.e. an autoimmune response). Remarkably, professional phagocytes of the innate immune system utilize a broad range of germ-line encoded receptors and opsonins to discriminate viable cells from pathogens and dying/dead cells, and aid the removal of non-self and altered-self via phagocytosis (Gregory and Devitt, 2004; Stuart and Ezekowitz, 2005). Not surprisingly, impairment of phagocytosis due to a deficiency in key phagocytic components such as C1q has been implicated in an increased susceptibility to *Streptococcus pyogenes* infection (Yuste et al., 2006) as well as in the development of autoimmune diseases such as SLE (Taylor et al., 2000). Therefore, understanding the molecular mechanisms of phagocytosis will provide new insights into numerous normal physiological and pathological processes.

Importantly, depending on the type of target cell (e.g. pathogens, apoptotic or necrotic cells), recognition and internalization by phagocytes can result in an anti- or a pro-inflammatory response (Stuart and Ezekowitz, 2005). Thus, the process of phagocytosis in mammals not only serves as an effector mechanism to clear infectious agents, dying/dead cells or unwanted materials, but also plays a critical role in determining the subsequent adaptive immune response towards the phagocytosed materials (Krysko et al., 2006) as well as orchestrating the process of wound healing and angiogenesis at sites of tissue injury (Tsioxianni et al., 2006). Furthermore, phagocytosis of dying/dead cells, especially apoptotic cells in an anti-inflammatory context, plays a vital role in maintaining immunological tolerance against cell-associated antigens (Tanaka et al., 2008). The second part of this chapter will only focus on (1) the complexity of phagocytosis of dying/dead cells, (2) the molecular mechanisms of dying/dead cells uptake, in particular the disposal of permeabilized/necrotic cells, (3) the striking similarities between pathogen and dying/dead cell removal, and (4) the immunological consequences of dying/dead cell clearance.
1.2.2 Recognition of target cells for phagocytosis

Although phagocytosis can simply be viewed as the engulfment of large particles (>0.5 μm) by cells via an actin-dependent mechanism, molecular events that govern different phases of phagocytosis are extremely complex (Aderem and Underhill, 1999) (Fig. 1.3). In order to mediate efficient removal of target cells, professional phagocytes such as macrophages, monocytes, neutrophils and dendritic cells (DCs) are located at high density in specific tissues (e.g. alveoli and spleen), where there is an increased chance of encountering target cells or being actively attracted to target cells via the so-called 'come-get-me' signals (Grimsley and Ravichandran, 2003). For example, generation of C5a molecules following complement activation on microbial surfaces can stimulate chemotaxis of neutrophils, macrophages and monocytes (Gerard and Gerard, 1994).

Figure 1.3. Schematic representation of the phases of phagocytosis and the recognition of target cells. The initial interaction between the phagocyte and the target cell involves adhesion molecules as well as recognition receptors that can detect various molecules exposed on the target cell either directly or indirectly via opsonins. The target cell may also release the so-called 'come-get-me' signals to attract phagocytic cells towards the site of homeostatic cell death, tissue injury or infection. Multiple interactions between the phagocytic cell and the target cell will lead to the formation a 'phagocytic synapse', which triggers various intracellular signalling events and induces the reorganization of the cytoskeleton to mediate target cell uptake. Following the engulfment process, the internalized target cell is delivered into the phagosome, which will mature into the phagolysosome for target cell killing and degradation. Phagocytic uptake of a target cell can trigger the release of anti-/pro-inflammatory cytokines, as well as the presentation of peptides derived from the target cell to T cells via both MHC class I and II molecules. Depending on the type of target cell, different levels and types of the so-called 'don't-eat-me' and 'eat-me' signals will be exposed. Viable cells expose self-associated molecular patterns (SAMPs) to prevent phagocytosis, whereas pathogens and dying cells (e.g. early apoptotic, late apoptotic and secondary necrotic cells) expose pathogen-associated molecular patterns (PAMPs) and apoptotic cell-associated molecular patterns (ACAMPs), respectively, to mediate their uptake by phagocytes. Note that permeabilized cells (e.g. primary necrotic, late apoptotic and secondary necrotic cells) and pathogens can also expose danger-associated molecular patterns (DAMPs), which are capable of stimulating phagocytes to promote a pro-inflammatory response.
Chapter 1

Initial interaction → Phagocytic synapse → Engulfment

**TARGET CELL**

Primary necrotic cell

Early apoptotic cell

Secondary necrotic cell

Pathogen

Viable cell

Early apoptotic cell

Late apoptotic cell

**Signals Exposed on the Target Cell**

- "Eat-me" signals
- PAMPs
- DAMPs
- ACAMPs
- DAMPs

**Signal Transduction Events**

- Cytoskeletal reorganization
- Anti/pro-inflammatory cytokines

**Soluble 'Come-get-me' signals**

- Adhesion molecules
- Opsonins

**Initial interaction**

**Phagocytic synapse**

**Engulfment**
Likewise, apoptotic cells can release chemotactic factors such as lysophosphatidylcholine (LPC) to attract phagocytes towards sites of apoptotic cell death (Lauber et al., 2003).

When a phagocyte comes into close proximity with a target cell, the target cell must expose a sufficient level of 'eat-me' signals to trigger phagocytosis. In order for the phagocyte to discriminate pathogens from host cells, Charles Janeway Jr proposed that a limited number of germ-line encoded pattern recognition molecules (PRMs) are used by the innate immune system to detect conserved molecular structures known as pathogen-associated molecular patterns (PAMPs) exposed on pathogens but absent on healthy host cells (Janeway, 1989) (Fig. 1.3). PRMs that are important in detecting PAMPs include Toll-like receptors (TLRs), the macrophage mannose receptor (MMR), macrophage scavenger receptors (MSR), C1q, MBL, CRP and serum amyloid protein (SAP), most of which recognize specific carbohydrate or polyanionic structures exposed on pathogens (Janeway and Medzhitov, 2002). Similarly, the ability of phagocytes to distinguish dying cells, such as apoptotic cells from viable cells, also relies on the exposure of the so-called apoptotic-cell-associated molecular patterns (ACAMPs) on apoptotic cells (Franc et al., 1999) (Fig. 1.3). However, due to the complexity of the molecular pathways that initiate cell death as well as the continuous progression through different stages of cell death (Fink and Cookson, 2005), it is difficult to use the term ACAMPs to describe molecular structures exposed by all dying/dead cells. To illustrate the complexity of signals exposed by self, altered-self and non-self, a number of selected examples will be discussed below and are schematically represented in Figure 1.4.

Besides insufficient levels of 'eat-me' signals on healthy host cells, viable cells can actively expose 'don't eat-me' signals (also known as self-associated molecular patterns (SAMPs)) to prevent their uptake by phagocytes (Elward and Gasque, 2003) (Fig. 1.3). Expression of CD47, a ubiquitous member of the Ig superfamily, on viable cells can deliver a negative engulfment signal through the immune inhibitory receptor SIPRα on macrophages (Oldenborg et al., 2001; Gardai et al., 2005; Tsai and Discher, 2008).
Figure 1. Complexity of signals exposed on target cells. Phagocytic cells are equipped with a variety of germ-line encoded detection mechanisms to distinguish self (viable cell), altered-self (apoptotic and necrotic cell) and non-self (pathogen) based on the combination of molecular signals exposed on the target cell. For example, viable cells expose a combination of "don’t-eat-me" signals such as CD47, CD31 and specific carbohydrate structures to prevent their uptake by phagocytes via a variety of mechanisms. When cells undergo apoptosis, various "eat-me" signals such as PS and DNA are exposed and several "don’t-eat-me" signals are lost or modified to formulate a specific combination of signals that enhances phagocytosis. When apoptotic cells become permeabilized, additional molecules (e.g. eIF3) are revealed to shape a new combination of "eat-me" and "don’t-eat-me" signals. Intracellular autoantigens can also be exposed once the cell membrane is permeabilized. If a different combination of signals is exposed by the permeabilized cell, phagocytic cells may not recognize the cell as apoptotic, and phagocytosis can lead to the exposure of modified molecules depending on the pathway of cell death. For example, primary necrotic cells will not expose molecules that are modified during apoptosis and thus reveal a different combination of signals compared to late apoptotic and secondary necrotic cells. Furthermore, pathogens can also expose a unique combination of signals (e.g. LTA, PCh, flagellin and CpGDNA) that can be recognized by phagocytes. It is important to acknowledge that the process of cell death is complex and can lead to the exposure of different combinations of signals depending on the pathway of cell death. For example, some combinations of signals may enhance phagocytosis, while others may inhibit it. A variety of soluble factors are also released by the target cell to prevent uptake (e.g. factor H), attract phagocytes (e.g. LPC) or directly modulate the immunological outcome of phagocytosis (e.g. HMGB1 and LPS).
Similarly, another member of the Ig superfamily, CD31 (PECAM-1), can also mediate the detachment of viable leukocytes from macrophages via homophilic interaction between CD31 expressed on both cell types (Brown et al., 2002). Furthermore, limiting activation of the complement cascade on viable cells by complement regulators such as CD46 (Elward et al., 2005) and factor H (Ollert et al., 1995) can prevent the recognition of viable cells by complement receptors (CR) expressed on various phagocytic cells. Intriguingly, although the function of the so-called anti-chemotactic factors in normal healthy tissues are not well understood (Fujita et al., 1997; Grutkoski et al., 2003), they may also participate in preventing the interaction between phagocytes and viable cells.

In contrast, when cells undergo apoptosis in response to either extrinsic or intrinsic mediators, a distinct set of morphological (e.g. cell shrinkage and blebbing) and biochemical (e.g. activation of caspases) changes occur (Fink and Cookson, 2005), which result in the release of ‘come-get-me’ signals (e.g. LPC), the exposure of ‘eat-me’ signals, as well as the loss of ‘don’t eat-me’ signals which collectively facilitate apoptotic cell clearance (Grimsley and Ravichandran, 2003). The loss of phospholipid asymmetry of the plasma membrane during the early stages of apoptosis can lead to the exposure of anionic phospholipids such as phosphatidylserine (PS), which can be recognized by a number of phagocytic receptors and opsonins such as PS receptor (Fadok et al., 2000) and scavenger receptors (e.g. LOX-1 and class B scavenger receptor type I) (Oka et al., 1998; Kawasaki et al., 2002), as well as the opsonins β2-glycoprotein I (β2GPI) (Maiti et al., 2008), milk fat globular-EGF factor 8 protein (MFG-E8) (Hanayama et al., 2002) and Protein S (Uehara and Shacter, 2008). It is also important to note that phagocytosis of target cells (e.g. apoptotic cells) is dependent on both the quantity and quality of positive (i.e. ‘eat-me’) and negative (i.e. don’t eat-me’) phagocytic signals. For example, ‘eat-me’ signals such as PS are not totally absent on viable cells, with a sufficient threshold level of externalized PS being required to initiate phagocytosis (Borisenko et al., 2003). In addition, activation of apoptotic pathways can lead to the modification or loss of ‘don’t eat-me’ signals such CD47 (Gardai et al., 2005), CD31 (Brown et al., 2002) and CD46 (Elward et al., 2005), which can further favour the uptake of apoptotic cells.
If apoptotic cells persist due to an overload of dying cells and/or an impairment in phagocytosis, early apoptotic cells will progress into late apoptotic and secondary necrotic cells, states in which the cell membrane becomes more permeable (Munoz et al., 2005). Similarly, generation of primary necrotic cells by extreme trauma can also result in the permeabilization of the cell membrane (Zhivotovsky and Orrenius, 2001). Importantly, the loss of membrane integrity exposes intracellular molecules that are previously hidden and may act as additional ‘eat-me’ signals to facilitate the clearance of permeabilized/necrotic cells. Although late apoptotic cells and primary/secondary necrotic cells are often grouped together simply as permeabilized cells (i.e. cells staining positive for PS-binding annexin V and the membrane impermeable DNA binding dye, propidium iodine), they are likely to expose different combinations of phagocytic signals due to differences in the molecular events that have occurred prior to membrane permeabilization (see Fig. 1.4). Whilst many of the intracellular ‘eat-me’ signals are not well characterized, a growing number of opsonins and receptors have been identified recently that can specifically aid the removal of permeabilized cells (discussed in detail in Section 1.2.3 below).

Similar to the ‘immunological synapse’ formed between a T cell and an antigen-presenting cell, the interaction between a phagocyte and a target cell also requires a combination of adhesion as well as recognition molecules to mediate the formation of a ‘phagocytic synapse’ (Stuart and Ezekowitz, 2005), which plays an important role in initiating various signalling events in the phagocyte to trigger cytoskeletal reorganization and the engulfment of the target cell via an actin-based mechanism (Aderem and Underhill, 1999) (Fig. 1.3). Following the engulfment process, the internalized target cell is delivered to a membrane-limited organelle known as the phagosome, which will eventually mature into a phagolysosome through a series of fusion and fission events with endosomes and lysosomes. Most importantly, phagocytic cells are equipped with the necessary molecular machinery to kill and degrade the internalized target cell, and subsequently process and present antigens derived from the target cell to the adaptive immune system via both MHC class I and II molecules (Stuart and Ezekowitz, 2005) (Fig. 1.3).
Furthermore, phagocytes can either direct an anti- or a pro-inflammatory response towards the site where phagocytosed material was identified depending on the type of immunomodulatory signals being released or exposed by the target cell. For example, bacteria can display exogenous 'danger' signals (also known as danger-associated molecular patterns (DAMPs) (Seong and Matzinger, 2004)), such as lipopolysaccharide (LPS), lipoteichoic acid (LTA), flagellin and peptidoglycan, which can activate TLRs and trigger the release of pro-inflammatory cytokines (Hallman et al., 2001). In contrast, uptake of apoptotic cells can inhibit the secretion of pro-inflammatory cytokines and promote the release anti-inflammatory cytokines (Fadok et al., 1998). Interestingly, permeabilized cells can also release endogenous 'danger' signals such as uric acid/monosodium urate (MSU) crystals (Shi et al., 2003) and high mobility group box 1 (HMGB1) molecules (Scaffidi et al., 2002) which can elicit a pro-inflammatory response. Therefore, the initial interaction between the phagocyte and the target cell may play a critical role in determining whether an immunogenic or a tolerogenic response will be raised towards the internalized material (discussed in detail in Section 1.2.5 below). Although a detailed discussion on how an immunogenic or a tolerogenic response is developed and maintained is beyond the scope of this literature review, it is important to note that the quality and the types of immune response generated towards the phagocytosed material is also influenced by a variety of factors and regulatory mechanisms present at different stages of the immune response (e.g. clonal selection, maturation of DCs and antigen presentation, T and B cell activation and proliferation, as well as regulation by T regulatory cells) (Stuart and Ezekowitz, 2005; Goodnow, 2007).

1.2.3 Molecular mechanisms of permeabilized/necrotic cell removal

According to the 'waste disposal' hypothesis (Walport, 2001) and the 'danger' hypothesis (Matzinger, 1994), massive apoptosis and/or a failure in dying/dead cell clearance can lead to the generation of permeabilized/necrotic cells and the exposure of immunostimulatory molecules as well as autoantigens to the immune system. Subsequently, phagocytic uptake of permeabilized/necrotic cells by antigen presenting cells, such as DCs, can lead to the presentation of autoantigens to autoreactive T cells in
a pro-inflammatory context, thus facilitating the onset of an autoimmune response. Since apoptotic cell removal is an important 'check-point' for autoimmunity, a substantial amount of effort in the last decade has been devoted to identifying the molecular mechanisms of apoptotic cell uptake and is extensively reviewed elsewhere (Elward and Gasque, 2003; Gregory and Devitt, 2004; Krysko et al., 2006). Recently, a number of studies have discovered several novel pathways of permeabilized/necrotic cell recognition and uptake (see Fig. 1.5 and below). A better understanding of the molecular mechanisms underpinning these pathways may shed light on the differences observed in the immunological outcome following the clearance of early apoptotic and permeabilized/necrotic cells by phagocytes.

1.2.3.1 Classical complement pathway

The complement system has been known to play a critical role in providing the first line of host defense against invading pathogens. Complement components can be activated on the surface of pathogens via three different pathways, the classical pathway, the lectin pathway, and the alternative pathway (Sim and Tsiftsoglou, 2004). Activation of the complement cascade serves three major roles, namely (1) inducing inflammation via fragments of complement components (e.g. C3a, C4a, C5a), (2) opsonization of pathogens for phagocytosis (e.g. via C3b), and (3) direct killing of pathogens through the assembly of the membrane-attack complex (MAC) (Sim and Tsiftsoglou, 2004). In addition, recent studies have implicated the complement system in dying/dead cells clearance, in particular the complement component C1q of the classical pathway.

C1q is a member of the collagen family and serves as a key PRM of the classical complement pathway (Sim and Tsiftsoglou, 2004). C1q interacts with pathogen surfaces either through direct interaction with charged/hydrophobic structures or indirectly via pathogen-binding IgG or IgM and subsequently promotes the activation of the serine proteases C1r and C1s (Sim and Tsiftsoglou, 2004). Initially C1q, as well as factor B, were identified as key components in human serum that can enhance the phagocytic
## Molecular mechanisms of apoptotic cell uptake

<table>
<thead>
<tr>
<th>Exposed ligand</th>
<th>Opsonin</th>
<th>Receptor</th>
<th>References</th>
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<tbody>
<tr>
<td>Nuclear structures/ phospholipids</td>
<td>CRP</td>
<td>FcγRI, FcγRIIA</td>
<td>Reviewed in (Marnell et al., 2005)</td>
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<tr>
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<td>HRG</td>
<td>FcγRI</td>
<td>(Gorgani et al., 2002) (Jones et al., 2005b)</td>
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<td>HSPG</td>
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<td>DNA</td>
<td>Ficolin 2, Ficolin 3</td>
<td>Calreticulin</td>
<td>(Kuraya et al., 2005) (Honore et al., 2007) (Jensen et al., 2007)</td>
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<tr>
<td>Nucleic acids/ phospholipids</td>
<td>MBL</td>
<td>Calreticulin</td>
<td>(Ogden et al., 2001) (Nauta et al., 2003)</td>
</tr>
<tr>
<td>DNA</td>
<td>PS, C1q</td>
<td>Calreticulin</td>
<td>Reviewed in (Trouw et al., 2008)</td>
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<tr>
<td>Autoantigens</td>
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<td>Calreticulin</td>
<td>(Shaw et al., 2000) (Kim et al., 2002) (Zwart et al., 2004) (Ciurana et al., 2004) (Quartier et al., 2005) (Ogden et al., 2005) (Fu et al., 2007)</td>
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<td>Autoantigens</td>
<td>IgG</td>
<td>Complement Receptors</td>
<td>(Ciurana et al., 2004) (Zwart et al., 2004) (Reefman et al., 2007) (Grossmayer et al., 2007)</td>
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<td>(Xu et al., 2008)</td>
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<td>?</td>
<td>TSP-1</td>
<td>Complement Receptors</td>
<td>(Savill et al., 1992) (Ren et al., 2001) (Krispin et al., 2006)</td>
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**Figure 1.5. Molecular mechanisms of necrotic cell clearance.** The interaction between phagocytes and necrotic cells involves a variety of receptors, opsonins and necrotic cell ligands as indicated in the figure. Besides the depicted phagocytic pathways, molecular mechanisms of apoptotic cell uptake are likely to work in concert with these pathways to aid necrotic cell removal. Note that CRP, SAP, HRG, Ficolin 2, Ficolin 3, MBL and C1q can all potentially modulate the activation of complement in addition to IgM, IgG and properdin to facilitate necrotic cell clearance. CRP, C-reactive protein; DNA, deoxyribonucleic acid; FcyR, Fcγ receptor; HERDS, heterogeneous ectopic ribonucleoprotein-derived structures; HRG, histidine-rich glycoprotein; HSPG, heparan sulfate proteoglycans; Ig, immunoglobulin; LPC; lysophosphatidylecholine; MBL, mannose-binding lectin; OxLDL, oxidized low-density lipoprotein; PS, phosphatidylserine; SAP, serum amyloid protein; TSP-1, thrombospondin-1.

uptake of apoptotic cells via CR3 and CR4 on macrophages, suggesting that the activation of both the classical and alternative pathways are required to aid apoptotic cell clearance (Mevorach et al., 1998). Subsequently it was shown, however, that C1q alone can bind directly to blebs on the surface of apoptotic cells (Ogden et al., 2001), possibly via PS (Mevorach et al., 1998) or DNA recognition (Uwatoko and Mannik, 1990), and augment apoptotic cell removal via calreticulin and CD91 on macrophages (Ogden et al., 2001). The importance of C1q and the classical pathway in apoptotic cell disposal was further demonstrated by the impairment of apoptotic cell uptake in C1q deficient mice in vivo as well as in C1q deficient patients in vitro (Taylor et al., 2000). Interestingly, complement binding and activation was later found to occur predominantly on subcellular fragments of late apoptotic and necrotic cells (Gaipl et al., 2001), and the deposition of C3 and C4 (critical components of the classical complement cascade) on dying/dead cells was also found to be highly dependent on C1q (Ciurana et al., 2004). Although phagocytic assays were not performed in these studies to validate the role of complement in late apoptotic and necrotic cell uptake, it is likely that complement may function mainly in the removal of permeabilized/necrotic cells rather than non-permeabilized early apoptotic cells. It is worth noting that the classical complement pathway can also be activated indirectly by necrotic cells via bridging molecules such as IgG, IgM, CRP and SAP (discussed in detail below).
Immunoglobulin/antibody plays a fundamental role in the adaptive immune response against foreign antigens (1) by forming immune complexes which neutralize and mediate the clearance of pathogen-derived toxins/substances, (2) by opsonizing pathogens to prevent further infection and facilitate their removal by professional phagocytes, and (3) by activating the classical complement pathway (Mix et al., 2006). In the event of autoimmunity, autoantibodies can also be induced against endogenous molecules, such as IgG in patients with rheumatoid arthritis (Dorner et al., 2004) and nuclear components in SLE patients (Gaipl et al., 2005). Interestingly, ‘natural’ IgM antibodies that bind specifically to structures exposed on apoptotic cells, such as LPC (Kim et al., 2002), oxidized LDL and phosphorylcholine (Shaw et al., 2000), have been proposed to play an important role in apoptotic cell removal via C1q binding and the activation of the classical complement pathway (Kim et al., 2002; Ogden et al., 2005; Quartier et al., 2005). Importantly, activation of complement on dying/dead cells is largely dependent on IgM (Kim et al., 2002; Ciurana et al., 2004; Zwart et al., 2004; Ogden et al., 2005; Quartier et al., 2005). In addition to the above mechanisms, IgM can potentially aid dying/dead cell clearance via Fc-α/μ receptor, which has been shown to mediate the endocytosis of IgM-coated microbes (Shibuya et al., 2000). Intriguingly, recent studies have shown that plasma IgM (Ciurana et al., 2004; Zwart et al., 2004), as well as the poly-reactive ‘natural’ IgM (3B4) (Fu et al., 2007), bind preferentially to permeabilized dying/dead cells and mediate complement activation. These studies are in agreement with others which show that complement binding occurs mainly on permeabilized late apoptotic and necrotic cells (Gaipl et al., 2001). Furthermore, it is worth noting that similar to C1q deficient mice, mice lacking serum IgM also develop lupus-like autoimmune diseases (Boes et al., 2000; Ehrenstein et al., 2000), which appears to be a result of an impairment in dying/dead cell clearance. In addition to IgM, plasma IgG (Ciurana et al., 2004; Zwart et al., 2004) as well as IgG autoantibodies from SLE patients (Grossmayer et al., 2007; Reefman et al., 2007), were found to bind largely to permeabilized dying/dead cells. However, the precise role of these ‘natural’ and
‘auto’ IgGs in the disposal of permeabilized dying/dead cells via CR and FcγR requires further investigations.

1.2.3.3 Mannose-binding lectin

MBL, a member of the collectin family, functions as a PRM that can recognize carbohydrates such as D-mannose and N-acetyl-D-glucosamine (GlcNAc) on microbial surfaces and mediate pathogen removal via the activation of the lectin complement pathway through MBL-associated serine protease (MASP) (Takahashi et al., 2006). Besides serving as a circulating innate defence protein against infectious agents, MBL has been shown recently to interact with a variety of endogenous ligands such as dying and damaged cells, immunoglobulins, nucleic acids and phospholipids (Takahashi et al., 2006). Similar to Clq, MBL was first demonstrated to bind specifically to apoptotic cells and facilitate their removal via calreticulin and CD91 on phagocytes (Ogden et al., 2001). However, later studies showed that MBL binds strongly to late apoptotic and necrotic cells, but not early apoptotic cells, via the lectin domain (Nauta et al., 2003). Interestingly, in contrast to the diffuse binding pattern of MBL on certain live cells, MBL was found to cluster on the surface of late apoptotic cells, suggesting that a clustering of MBL on dying/dead cell surfaces is required for MBL to trigger phagocytosis (Stuart et al., 2005). Although both Clq and MBL compete for the same or adjacent structures on late apoptotic cells (Nauta et al., 2003), Clq may dominate over MBL in enhancing dying/dead cell clearance as the concentration of circulating Clq in human plasma is usually approximately 100-fold higher than that of MBL. Indeed, in vivo studies demonstrated that MBL deficient mice have impaired late apoptotic cell clearance but do not develop severe autoimmunity like Clq deficient mice (Stuart et al., 2005). Nevertheless, since MBL has been suggested to function as an acute-phase protein (Ezekowtiz et al., 1988; Arai et al., 1993), MBL may play an important role in dying/dead cell removal during the acute phase response.
1.2.3.4  Ficolin-2 and Ficolin-3

Similar to MBL, other serum lectins such as Ficolin-2 (L-Ficolin) and Ficolin-3 (H-Ficolin) have also been shown to mediate the clearance of dying/dead cells (Kuraya et al., 2005; Honore et al., 2007; Jensen et al., 2007). In humans three kinds of ficolin have been identified (Ficolin-1, -2 and -3) which exhibit both structural and functional similarities to MBL and C1q (Endo et al., 2007). Like MBL, ficolins can recognize specific carbohydrate structures, such as GlcNAc exposed on the surface of pathogens, and activate the lectin complement pathway through association with MASP (Endo et al., 2007). Consistent with earlier studies by Kuraya et al. (2005), both Ficolin-2 (Jensen et al., 2007) and Ficolin-3 (Honore et al., 2007) are able to bind to dying/dead cells, in particular permeabilized cells, and function as opsonins to aid phagocytic uptake. Furthermore, Ficolin-2 was also found to be an important serum factor in activating complement on dying/dead cells (Kuraya et al., 2005; Jensen et al., 2007). Although it was proposed that both Ficolin-2 and Ficolin-3 may act as adaptor molecules between exposed DNA on late apoptotic/necrotic cells (Honore et al., 2007; Jensen et al., 2007) and calreticulin on phagocytes (Kuraya et al., 2005; Honore et al., 2007), further studies (e.g. using DNase treated cells and appropriate blocking antibodies) are necessary to elucidate the underlying mechanisms.

1.2.3.5  Properdin

Besides the components of the classical and the lectin complement pathways, properdin, the only known positive regulator of complement activation, has been shown recently to mediate activation of the alternative complement pathway on dying/dead cells (Kemper et al., 2008; Xu et al., 2008). Properdin, a single chained glycoprotein that is present in plasma as cyclic polymers, was proposed to play an important role in the enhancement of complement activation through its interaction with C3b and stabilization of the C3 and C5 convertase complexes (Schwaeble and Reid, 1999). Although properdin is only present at the relatively low concentration of ~5-15 µg/ml in plasma, it can be stored in neutrophil granules and released at sites of inflammation (Schwaeble and Reid, 1999).
addition to binding C3b coated surfaces, recent studies have also demonstrated that
properdin can initiate complement activation via direct interaction with microbial
surfaces (Spitzer et al., 2007). Interestingly, studies by Xu et al. (2008) showed that
properdin binds exclusively to late apoptotic and necrotic cells independently of C3b,
possible through the recognition of exposed DNA at the later stages of cell death.
Importantly, once bound properdin activates complements on necrotic cells via the
alternative pathway (Xu et al., 2008). Although conflicting studies have reported that
properdin binds predominately to early apoptotic cells and aids dying/dead cell uptake
by phagocytes, either in the presence or absence of complement activation (Kemper et
al., 2008), it has been speculated that properdin may also play a critical role in
regulating complement activation on late apoptotic and necrotic cells (Xu et al., 2008).

1.2.3.6  Pentraxin family

Pentraxins are a superfamily of proteins characterized by the presence a 200 amino acid
pentraxin domain at the C-terminus (Garlanda et al., 2005). Pentraxins can be further
subdivided into the classic short pentraxins like CRP and SAP, and long pentraxins such
as pentraxin-3 (PTX3) (Garlanda et al., 2005). In humans, CRP is a major acute-phase
plasma protein, in which the serum concentration can rapidly increase up to 1000-fold in
response to infection or tissue injury (Volanakis, 2001). Being one of the first innate
immunity molecules identified, it is well known that CRP binds to phosphocholine
(PCh). PCh is a major constituent of many bacterial and fungal polysaccharides and
most biological cell membrane, and CRP aids the removal of pathogens by acting as an
adaptor molecule that binds PCh and activates the classical complement pathway
through its interaction with C1q (Marnell et al., 2005). Although the literature regarding
the interaction between CRP and various FcγR has been controversial, due to IgG
contaminating CRP preparations (Hundt et al., 2001) and concerns regarding the
techniques used to detect CRP binding to FcγRIIA (Saeland et al., 2001), numerous
studies have suggested that CRP can act as an opsonin by binding to FcγR (Marnell et
al., 2005). In addition to the elimination of pathogens, studies by Gershov et al. (2000)
demonstrated that CRP can also bind to apoptotic cells in a Ca^{2+}-dependent manner and
enhance apoptotic cells uptake by macrophages when C1q containing serum is present. The authors also showed that the binding of CRP to apoptotic cells enhanced the activation of the classical complement pathway but prevented the assembly of the MAC (Gershov et al., 2000). Similarly, CRP has also been proposed to mediate the removal of apoptotic cells via a FcγR-dependent mechanism, based on studies using macrophages from Fc receptor common γ-chain deficient mice (Mold et al., 2002). Interestingly, recent studies showed that CRP (with low levels of contaminating IgG) binds exclusively to permeabilized late apoptotic neutrophils (Hart et al., 2005), possibly to nuclear structures (Pepys et al., 1994) or phospholipids (Li et al., 1994), but had no apparent effect on the phagocytosis of dying/dead cells in the absence of serum (Hart et al., 2005). Thus, it is possible that CRP may function predominantly via the classical complement pathway to aid the clearance of dying/dead cells, especially late apoptotic and necrotic cells, with the role of FcγR in this process still being controversial.

SAP, another classic short pentraxin, is constitutively present in human serum at ~30-40 μg/ml and represents a major acute phase reactant in mice (Garlanda et al., 2005). Like other pentraxins, SAP was found to bind complement components, bacteria, various carbohydrate determinants, as well as viruses (Garlanda et al., 2005). Interestingly, SAP can also bind chromatin subunits (Butler et al., 1990) and DNA (Pepys and Butler, 1987). Similar to CRP, SAP can interact with various FcγR (Bharadwaj et al., 2001) and mediate the uptake of apoptotic cells via a FcγR-dependent mechanism (Mold et al., 2002). However, later studies demonstrated that SAP binds predominantly to permeabilized late apoptotic cells and aids the phagocytosis of late apoptotic, but not early apoptotic, cells in phagocytic assays containing serum (Bijl et al., 2003). Thus, SAP can potentially mediate the uptake of permeabilized cells via both complement- and FcγR-dependent pathways.

The long pentraxin, PTX3, is a cytokine-inducible innate defence molecule that recognizes microbes and activates the classical complement pathway to enable the removal of pathogens (Garlanda et al., 2005). PTX3 binds specifically to permeabilized dying cells that have undergone apoptotic cell death but not to permeabilized/necrotic
cells generated by chemical, extreme temperature or mechanical treatments (Rovere et al., 2000). Paradoxically, PTX3 inhibits the uptake of late apoptotic cells by DCs (Rovere et al., 2000) and dampens the ability of DCs to cross-present antigens derived from the ingested apoptotic cells, which prevents the activation of autoreactive T cells (Baruah et al., 2006a; Baruah et al., 2006b). Furthermore, PTX3 was found to reduce C1q- (Baruah et al., 2006a) and SAP-mediated (van Rossum et al., 2004) phagocytosis of dying cells. Therefore, it is of great interest to investigate whether PTX3 deficient animals are more efficient in the disposal of permeabilized/necrotic cells and less prone to autoimmunity caused by an overload of apoptotic cells.

**1.2.3.7 Histidine-rich glycoprotein**

HRG, a member of the cystatin supergene-family, is an abundant multifunctional protein that is present in the plasma of many vertebrates (Jones et al., 2005a). HRG has a multidomain structure that allows the molecule to interact with many ligands including heparin, HS, plasminogen, fibrinogen, TSP, IgG, FcγR, C1q, haem and Zn²⁺ (Jones et al., 2005a). The ability of HRG to interact with various ligands simultaneously has suggested that HRG can act as an adaptor molecule and regulate numerous biological processes such as immune complex and pathogen clearance, angiogenesis, cell adhesion, coagulation and fibrinolysis (see Section 1.1). In addition, studies by Gorgani et al. (2002) showed that HRG binds strongly to late apoptotic cells compared to viable or early apoptotic cells, possibly by recognizing naked DNA exposed during apoptosis. Furthermore, HRG binds FcγRI on HMDM and functions as a bridging molecule to enhance the uptake of apoptotic cells via a FcγRI-dependent mechanism (Gorgani et al., 2002). Similarly, studies by Jones et al. (2005b) also demonstrated that, besides cell surface HS, HRG binds strongly, via its N-terminal domains, to cytoplasmic ligand(s) exposed on permeabilized/necrotic cells. However, HRG-mediated necrotic cell uptake was found to be independent of cell surface HS, FcγRI and FcγRIIA on phagocytes (Jones et al., 2005b). Thus, the molecular mechanisms underpinning the enhanced clearance of necrotic cells by HRG remain unknown.
1.2.3.8 Thrombospondin-1

Similar to HRG, TSP-1 is a multifunctional homotrimeric ECM protein that has been implicated in regulating a variety of biological processes including angiogenesis, chemotaxis, cell adhesion, cell proliferation and apoptosis (Sid et al., 2004). TSP-1 has a modular and multidomain structure that allows it to interact with a variety of ligands and receptors such as heparin, Ca$^{2+}$, integrins, CD36, CD47, fibronectin, latent TGF-β1 and several proteases (Sid et al., 2004). Interestingly, TSP-1 can also bind directly to peptidoglycan on gram-positive pathogens and aids their adherence to host cells (Rennemeyer et al., 2007). Although TSP-1 was originally characterized as an α-granule glycoprotein in platelets, it can be synthesized and released by a variety of cell types including epithelial and mesenchymatous cells (Sid et al., 2004), as well as macrophages (Savill et al., 1992) and apoptotic cells (Moodley et al., 2003; Krispin et al., 2006). Importantly, studies by Savill et al. (1992) demonstrated that TSP-1 plays a vital role in the phagocytosis of intact aging human neutrophils (PMNs) undergoing apoptosis, possibly by acting as a molecular bridge between late apoptotic cells and CD36 and the αvβ3 integrin (vitronectin receptor) on macrophages (Savill et al., 1992; Ren et al., 2001). Whilst the apoptotic cell ligand(s) of TSP-1 remains elusive, recent studies suggested that TSP-1 can bind strongly to permeabilized cells (Krispin et al., 2006) and work in concert with a PS-dependent mechanism to aid the clearance of necrotic cells (Bottcher et al., 2006).

1.2.3.9 Heparan sulfate proteoglycans

Heparan sulfate proteoglycans (HSPG) are composed of a protein core and side-chains of the complex glycosaminoglycan, HS (Tumova et al., 2000). HSPG can be found within the extracellular milieu of a variety of cells and tissues and have been implicated in numerous biological processes including the organization of the ECM as well as cell adhesion, migration, proliferation and differentiation (Tumova et al., 2000). The ability of HSPG to regulate various processes often depends on the interaction between the HS component of HSPG and a range of protein ligands such as growth factors, cytokines,
chemokines, enzymes and cell-adhesion molecules (Parish, 2006). Furthermore, studies have also demonstrated that HSPG can mediate the phagocytosis of latex beads via an actin-dependent mechanism (Fukasawa et al., 1997; Dehio et al., 1998). Interestingly, invasive bacteria such as Neisseria gonorrhoeae can hijack the HSPG-dependent phagocytic pathway to facilitate their entry into host epithelial cells via the interaction between HSPG and the pathogen-derived opacity-associated protein (Naumann et al., 1999). Recently, studies by Gebska et al. (2002) showed that heparin, essentially a more sulfated form of the HS side-chains of HSPG, can bind strongly to dying cells compared to viable cells, especially secondary necrotic cells. Confocal microscopy studies further indicated that heparin may bind to nuclear materials known as heterogeneous ectopic ribonucleoprotein-derived structures (HERDS) (Gebska et al., 2002). Based on the ability of pentosan polysulfate to block heparin binding sites on dead cells and inhibit the phagocytosis of dead cells, the authors suggested that HSPG on macrophages may play an important role in dying/dead cell clearance (Gebska et al., 2002). However, further studies are necessary to provide direct evidence that HSPG play a role in mediating necrotic cell removal.

1.2.3.10 Molecular mechanisms of apoptotic cell clearance

Since necrotic cells are permeabilized and may originate from uncleared apoptotic cells, it is not surprising that many of the molecular mechanisms involved in apoptotic cell clearance are also involved in the phagocytosis of necrotic cells. For example, the well-characterized ‘eat-me’ signal PS on apoptotic cells (see Section 1.2.2) was shown recently to promote the recognition and clearance of permeabilized/necrotic cells (Hirt and Leist, 2003; Brouckaert et al., 2004; Bottcher et al., 2006). Similarly, the glycosylphosphatidylinositol-linked plasma-membrane glycoprotein CD14, a PRM that binds the LPS-binding protein (LBP)-LPS complex (Bosshart and Heinzelmann, 2007), was found to be an important receptor for both apoptotic (Devitt et al., 1998; Devitt et al., 2003; Devitt et al., 2004) and necrotic cell uptake (Bottcher et al., 2006), possibly by binding to the modified ICAM-3 exposed on dying/dead cells (Moffatt et al., 1999). Interestingly, CD14 can also enhance the phagocytosis of LBP-opsonized gram-negative
bacteria such as *Escherichia coli* (Schiff et al., 1997). Thus, similar to various permeabilized/necrotic cell-specific removal pathways described above, CD14 can aid the uptake of both pathogens and dying/dead cells.

### 1.2.4 Similarities between pathogen and necrotic cell recognition and phagocytosis

As more phagocytic pathways are being identified, it is becoming clear that the same germ-line encoded PRMs such as C1q, MBL, CRP, SAP and CD14 are used by the innate immune system to detect both pathogens and dying/dead cells (see Section 1.2.3 above). Although utilizing the same molecular mechanisms may provide an evolutionary advantage in efficiently removing both ‘non-self’ and ‘altered-self’ materials that may cause harm to the organism, it is unclear whether one function came before the other or whether both functions co-evoluted together. Admittedly, the literature was at first somewhat puzzling, it appearing that soluble opsonins that were well known to initiate a pro-inflammatory response against ‘danger-associated’ pathogens were also involved in the removal of ‘danger-free’ early apoptotic cells. This suggested that a common recognition pathway was used to detect two very distinct target cells but promote opposing immunological outcomes. Intriguingly, many of these PRMs have recently been demonstrated to aid the uptake of ‘danger-associated’ permeabilized late apoptotic/necrotic cells but not early apoptotic cells (see Section 1.2.3 above). Thus, the innate immune system may have evolved separate mechanisms to recognize and remove early apoptotic cells prior to cell lysis to avoid inflammation and maintain self tolerance, but was also pressured to evolve additional PRMs to detect molecular structures that are exposed on permeabilized cells as well as pathogens, and alert the immune system to potential danger. These additional PRMs may also function as a backup mechanism to efficiently remove permeabilized cells and prevent further tissue damage. It is worth noting that these innate recognition mechanisms are likely to have predated the evolution of the adaptive immune system. How these phagocytic pathways modulate the immunological response to permeabilized/necrotic cells in higher organisms will be discussed below.
1.2.5 Immunological consequence of permeabilized/necrotic cell clearance

The process of phagocytosis not only serves as an effector mechanism to eliminate pathogens and dying/dead cells, it also plays a vital role in orchestrating the subsequent immune response towards the phagocytosed materials. Although apoptotic cell uptake is often considered to be anti-inflammatory and induces a tolerogenic response, whereas necrotic cell removal is associated with inflammation and promotes immunity/autoimmunity, factors that determine the immunological consequences of apoptotic and necrotic cell clearance are extremely complex and sometimes controversial. As summarized in Figure 1.6, the immunological outcome of dying/dead cell removal is influenced by multiple factors such as the types of cells undergoing cell death, the stages of cell death, the recognition and uptake pathways used by the phagocytes, the types of phagocytes, as well as the location and microenvironment of cell death. Due to this complexity, contrasting results have been reported in the literature (Krysko and Vandenabeele, 2008), possibly due to differences in the experimental models being used. Although the origin of the dying/dead cell (e.g. lymphocyte or infected cell) may influence the types of cell-associated antigens (e.g. viral proteins in the case of virus infection) being revealed to the immune system, the combination of ‘danger’ and ‘phagocytic’ signals being exposed by the dying/dead cell and the activation status of the different types of phagocytic cells involved in dying/dead cell clearance are key factors in determining the quality and the types of immune response (see below).

1.2.5.1 Danger signals

Membrane integrity of dying cells has been used as one of the most important criteria to differentiate different stages of cell death (e.g. early apoptotic versus late apoptotic cells). Functionally, the loss of membrane integrity in late apoptotic and primary/secondary necrotic cells also plays a critical role in the release of molecules known as ‘danger’ signals or DAMPs that can induce a pro-inflammatory response via a range of different mechanisms (Kono and Rock, 2008) (see also Section 1.2.2). The Danger
Different antigens can be exposed to the immune system depending on the types of cells that are undergoing cell death (e.g. pancreatic islet cells, leukocytes, lymphocytes, thymic cells or bacteria/virus infected cells).

Different molecules can be exposed to the immune system depending on the stages of cell death (e.g. early apoptotic, late apoptotic, secondary necrotic or primary necrotic).

The immune system can respond differently to dying/dead cells depending on the location of dying/dead cell (e.g. blood, spleen, liver or the primary site of cell death) and the specific microenvironment at sites of cell death (e.g. the presence of various cytokines and 'danger' signals).

Different recognition and uptake pathways can be utilized depending on the type of phagocyte (e.g. monocyte, macrophage, dendritic cell or neutrophil) and their activation status, as well as the availability of various opsonins.

**Figure 1.6. Factors that determine the immunological outcome of dying/dead cell clearance.** The immunological consequences of dying/dead cell removal can be modulated by a variety of factors including the types of cells undergoing cell death, the stages of cell death, the recognition and uptake pathways used by the phagocytes, the types of phagocytes, as well as the location and microenvironment of cell death.

Model, originally proposed by Polly Matzinger, suggests that the immune system has evolved to focus on and respond to materials that are potentially dangerous, rather than on those that are simply foreign or infectious (Matzinger, 1994). Initially, apart from various pathogens, dying and damaged host cells were also found to directly induce an immunogenic response in the absence of exogenous adjuvants (Gallucci et al., 1999; Shi et al., 2000). Importantly, the immunostimulatory activity of injured cells was found to be constitutively present mainly in the cytoplasm but not in the nucleus of cells, and can increase when certain apoptotic pathways are activated (Shi et al., 2000). As described earlier, a number of endogenous ‘danger’ signals have been discovered (see Kono and...
Rock (2008) for an extensive review), including uric acid/MSU crystals (Shi et al., 2003), heat shock proteins (HSP) (Basu et al., 2000), HMGB1 (Scaffidi et al., 2002) and double stranded (ds) DNA (Ishii et al., 2001). For example, HMGB1, an abundant chromatin-binding protein, can be dissociated from the nucleus and release by necrotic cells to trigger a pro-inflammatory response (Scaffidi et al., 2002). Although the molecular mechanisms underpinning the inflammatory effect of HMGB1 is controversial, it was reported recently that HMGB1 could form complexes with DNA or DNA-containing immune complexes and stimulate the production of cytokines via a TLR-9- and receptor for advanced glycation end-products (RAGE)-dependent pathway (Tian et al., 2007).

Besides necrotic cells, apoptotic cells can also expose various immunostimulatory signals. For example, the chemoattractant LPC, which is released by early apoptotic cells (Lauber et al., 2003) (see Section 1.2.2), can also function as a pro-inflammatory signal to induce the production of pro-inflammatory cytokines such as macrophage-inflammatory protein-2 (MIP-2) (Olofsson et al., 2008) and promote the maturation of DCs (Coutant et al., 2002). Similarly, exposure of nucleic acids on the surface of early apoptotic cells (Elward et al., 2005) can potentially deliver a ‘danger’ signal to phagocytes via TLR-9 following apoptotic cell uptake. Furthermore, it has also been reported that HMGB1 can be released by cells undergoing apoptotic cell death (Bell et al., 2006). Thus, the exposure of ‘danger’ signals is not an exclusive event associated with necrotic cells, which further complicates predicting the immunological consequences of dying/dead cell removal.

1.2.5.2 Recognition and uptake pathways

Although the initial ‘danger’ signals released by dying/dead cells are important in stimulating phagocytic cells, the subsequent interaction between a phagocyte and a dying/dead cell via various receptors and opsonins would be expected to provide additional signals that modulate the immune response. For example, the binding of C1q and MBL by human monocytes was found to suppress the secretion of LPS-induced pro-
inflammatory cytokines like IL-1α and IL-1β, and promote the secretion of cytokines such as IL-10, IL-6 and monocyte chemoattractant protein-1 (MCP-1) (Fraser et al., 2006). Although it has been reported that a combination of LPS and C1q can induce the production of IL-12p70 by DCs (Baruah et al., 2006a), a potent pro-inflammatory cytokine required for T cell priming (Trinchieri, 1995), uptake of apoptotic cells via a C1q-dependent mechanism by DCs promoted the production of IL-6, IL-10 and TNF-α, but not IL-12p70 (Nauta et al., 2004). Thus, the clearance of permeabilized/necrotic cells via complement components such as C1q and MBL (see Section 1.2.3.1 and 1.2.3.3) may recruit additional phagocytes to survey sites of tissue injury and modulate the quality of the immune response. Furthermore, phagocytosis of permeabilized/necrotic cells via a PS-dependent pathway may also favour a non- or anti-inflammatory response (Hirt and Leist, 2003; Brouckaert et al., 2004; Bottcher et al., 2006). In contrast, clearance of dying/dead cells via an IgG-FcγR-dependent pathway may induce inflammation. For example, phagocytosis of β2GPI and anti-β2GPI mAb-opsonized apoptotic cells by DCs was found to enhance the production of IL-1β, TNF-α and IL-10, as well as the presentation of intracellular antigens to MHC class II-restricted CD4+ T cells (Rovere et al., 1999). Similarly, immune complexes binding to FcγRIIA on apoptotic neutrophils also promoted neutrophil clearance and the secretion of pro-inflammatory cytokines such as TNF-α and IL-6 by macrophages (Hart et al., 2004). Therefore, opsonization of permeabilized/necrotic cells by IgG (see Section 1.2.3.2) can potentially cross-link FcγR and induce the production of pro-inflammatory cytokines as well as antigen presentation.

Importantly, the receptors and opsonins used to recognize dying/dead cells depend mainly on the phagocytic cell type as well as their tissue location. For example, different CR and FcγR are differentially expressed among various phagocytes (e.g. neutrophils, monocytes, and subsets of macrophages and DCs) and the expression of these receptors may also vary depending on the activation state of the phagocytic cells (van de Winkel and Anderson, 1991; Bajtay et al., 2006; van Lookeren Campagne et al., 2007). Similarly, delivery of 'eat-me' signals via the CD14-dependent pathway may be limited solely to monocytes and macrophages (Gregory, 2000). To further highlight the
complexity of these recognition pathways, the relative importance of different mechanism of uptake at different locations/organs may also vary. For example, although macrophages derived from both scavenger receptor class A (SR-A) and CD14 deficient mice showed impairment in the removal of apoptotic cells in vitro (Platt et al., 1996; Devitt et al., 2004), CD14 but not SR-A deficient mice were defective in removing apoptotic cells in the thymus (Platt et al., 2000; Devitt et al., 2004), indicating that the phagocytic function of SR-A, but not CD14, is redundant in the thymus. Similarly, the clearance of apoptotic cells via MFG-E8 was found to be an important pathway in splenic and lymph node germinal centres but not in the thymus (Hanayama et al., 2004).

1.2.6 Concluding remarks

The uptake of dying/dead cells has been shown to play a critical role in tissue homeostasis, as well as providing a source of cell-associated antigens and immunomodulatory signals for immune tolerance/activation and regulate the quality of the immune response. Since necrotic cells are generally considered as activators of the immune system, elucidating the molecular mechanisms underpinning necrotic cell clearance may have important implications for understanding the initiation and maintenance of autoimmune disease such as SLE, as well as the development of anti-cancer treatment by enhancing the immunogenicity of cancer cells via the uptake of necrotic cancer cells by phagocytes, such as DCs, in a pro-inflammatory context.
Chapter Two

MATERIALS AND METHODS

This chapter details the materials and methods used for experiments described in this thesis.
2.1 General reagents

Details of general reagents, kits, medium and buffers used throughout this thesis are listed in Table 2.1.

2.2 Cell culture

2.2.1 Subculturing mammalian cell lines

Different mammalian cell lines were cultured in specific medium listed in Table 2.2 and incubated at 37°C in a Hepa-Filtered IR Incubator (Forma Scientific, Marietta, OH) that maintained a humidified atmosphere containing 5% CO₂. Cells were routinely passaged every two to three days according to the rate of cell proliferation. For non-adherent cells, cells were collected via centrifugation at 300 g for 5 mins, subcultured at a concentration of ~2 x 10⁴ cells/ml and allowed to grow to a maximum density of ~1 x 10⁶ cell/ml. For adherent cells, culture media was removed and the adherent cells washed with sterile PBS. To detach the adherent cells and avoid proteolytic cleavage of cell surface receptors, EDTA/PBS (in the absence of trypsin) was added and the cells incubated at 37°C for 5 to 10 mins in a 5% CO₂ atmosphere. Detached cells were collected, pelleted by centrifugation at 300 g for 5 mins and subcultured at an appropriate density.

2.2.2 Cell counting and viability

A 10 µl cell suspension from a known volume was diluted 1:1 with 0.1% Trypan Blue (BDH Poole Chemicals, London, England) and loaded onto a haemocytometer. Cells were then viewed at x40 magnification under a standard Olympus B light microscope (Olympus Optical, Tokyo, Japan) to assess cell number and viability.
### Table 2.1. General reagents, Kits, medium and buffers used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Details</th>
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<tr>
<td><strong>General reagents</strong></td>
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<tr>
<td>ABTS</td>
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<td>Alkaline phosphatase (calf intestinal)</td>
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<td>Aprotinin</td>
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<td>BSA (fatty acid free)</td>
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<tr>
<td>Name</td>
<td>Details</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>-------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>General reagents</strong></td>
<td></td>
</tr>
<tr>
<td>Human normal IgG</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Human plasmin</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Human plasminogen</td>
<td>Purified by Dr. Allison Jones as previously described (Moroi and Aoki, 1976: Rickli and Otavsky, 1975; Wiman, 1980)</td>
</tr>
<tr>
<td>Membrane Lipid Strips™</td>
<td>Echelon Biosciences, Salt Lake City, UT</td>
</tr>
<tr>
<td>NHS-activated Sepharose® 4 Fast Flow</td>
<td>Amersham Pharmacia Biotech, Amersham, Sweden</td>
</tr>
<tr>
<td>N-(p-Tosyl)-Gly-Pro-Lys</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Phosphatidic acid (synthetic)</td>
<td>Avanti Polar Lipids, Alabaster, AL</td>
</tr>
<tr>
<td>Phosphatidylinositol (bovine liver)</td>
<td>Avanti Polar Lipids, Alabaster, AL</td>
</tr>
<tr>
<td>Phosphatidylinositol-4-phosphate (porcine brain)</td>
<td>Avanti Polar Lipids, Alabaster, AL</td>
</tr>
<tr>
<td>Phosphatidylinositol-4,5-bisphosphate (porcine brain)</td>
<td>Avanti Polar Lipids, Alabaster, AL</td>
</tr>
<tr>
<td>Phospholipase C (Bacillus cereus)</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>PIP Strips™</td>
<td>Echelon Biosciences, Salt Lake City, UT</td>
</tr>
<tr>
<td>PKH26</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td>Protease inhibitor cocktail tablets</td>
<td>Roche Diagnostics, Mannheim, Germany</td>
</tr>
<tr>
<td>PSN (x1000)</td>
<td>Penicillin G 30 g/l, streptomycin sulfate 50 g/l, neomycin sulfate 50 g/l</td>
</tr>
<tr>
<td>Skim milk powder</td>
<td>Diploma, New Zealand</td>
</tr>
<tr>
<td>SphingoStrips™</td>
<td>Echelon Biosciences, Salt Lake City, UT</td>
</tr>
<tr>
<td>Sulfatide (porcine brain)</td>
<td>Avanti Polar Lipids, Alabaster, AL</td>
</tr>
<tr>
<td>Triton X-100</td>
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<tr>
<td>Tween-20</td>
<td>Sigma-Aldrich, St. Louis, MO</td>
</tr>
<tr>
<td><strong>Kits</strong></td>
<td></td>
</tr>
<tr>
<td>BD™ Cytometric Bead Array Human Inflammation Kit</td>
<td>BD Bioscience, San Jose, CA</td>
</tr>
<tr>
<td>Human IgG Subclass Profile ELISA Kit</td>
<td>Zymed Laboratories, San Francisco, CA</td>
</tr>
<tr>
<td>ImmunoPure® IgG1 Fab and F(ab')2 Preparation Kit</td>
<td>Pierce Biotechnology, Rockford, IL</td>
</tr>
<tr>
<td>Name</td>
<td>Details</td>
</tr>
<tr>
<td>----------------------</td>
<td>--------------------------------------------------------------------------</td>
</tr>
<tr>
<td>DMEM H16 medium</td>
<td>H16 powder 9.99 g/l (Gibco BRL, Grand Island, NY), NaHCO₃ 3.7 g/l</td>
</tr>
<tr>
<td>EDTA/PBS</td>
<td>PBS supplemented with 0.5 mg/ml EDTA</td>
</tr>
<tr>
<td>Ham's F-12 medium</td>
<td>Gibco BRL, Grand Island, NY</td>
</tr>
<tr>
<td>PBS</td>
<td>NaCl 8 g/l, Na₂HPO₄·2H₂O 1.25 g/l, NaH₂PO₄·H₂O 0.35 g/l</td>
</tr>
</tbody>
</table>

ABTS, 2,2'-azido-bis(3-ethylbenzthiazoline-6-sulphonic acid; BSA, bovine serum albumin; CFSE, Carboxy-fluorescin diacetate succinimidyl ester; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; Ig, immunoglobulin; PBS, phosphate buffered saline

Table 2.2. Details of cell lines, medium and supplements used in this study.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Details</th>
<th>Medium and supplements</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO-K1</td>
<td>Chinese hamster ovary cells (adherent)</td>
<td>50% DMEM and 50% Ham's F12, 10% FCS, PSN</td>
</tr>
<tr>
<td>pgsA-745</td>
<td>Chinese hamster ovary cells, xylosyltransferase deficient (adherent)</td>
<td>50% DMEM and 50% Ham's F12, 10% FCS, PSN</td>
</tr>
<tr>
<td>FoýR₁IA-arg131 pgsA-745</td>
<td>Chinese hamster ovary cells, xylosyltransferase deficient, stably expressing human FoýR₁IA-arg131 (adherent)</td>
<td>50% DMEM and 50% Ham's F12, 10% FCS, PSN</td>
</tr>
<tr>
<td>FoýR₁IA-his131 pgsA-745</td>
<td>Chinese hamster ovary cells, xylosyltransferase deficient, stably expressing human FoýR₁IA-his131 (adherent)</td>
<td>50% DMEM and 50% Ham's F12, 10% FCS, PSN</td>
</tr>
<tr>
<td>Jurkat T cell</td>
<td>Human leukemic T cells (non-adherent)</td>
<td>RPMI-1640, 5-10% FCS, PSN</td>
</tr>
<tr>
<td>THP-1</td>
<td>Human monocytic cells (non-adherent)</td>
<td>RPMI-1640, 10% FCS, PSN</td>
</tr>
<tr>
<td>EL4</td>
<td>Mouse thymoma cells (non-adherent)</td>
<td>RPMI-1640, 10% FCS, PSN</td>
</tr>
<tr>
<td>MS</td>
<td>Mouse L cells, fibroblast, transfected with DNA vector alone (adherent)</td>
<td>DMEM, 10% FCS, PSN, 3.2 μM Methotrexate</td>
</tr>
<tr>
<td>MS9-II</td>
<td>Mouse L cells, fibroblast, stably expressing human CIMPR (adherent)</td>
<td>DMEM, 10% FCS, PSN, 3.2 μM Methotrexate</td>
</tr>
<tr>
<td>MM170</td>
<td>Human melanoma cells (adherent)</td>
<td>RPMI-1640, 10% FCS, PSN</td>
</tr>
</tbody>
</table>

PSN represents a final concentration of penicillin G 30 μg/ml, streptomycin sulfate 50 μg/ml and neomycin sulfate 50 μg/ml. CIMPR, cation-independent mannose-6-phosphate receptor; DMEM, Dulbecco’s modified Eagle’s medium; DNA, deoxyribonucleic acid; FoýR, Fcγ receptor; FCS, fetal calf serum
2.2.3 Cyropreservation of mammalian cell lines

Cells were collected as described in Section 2.2.1 and resuspended at a cell concentration of \( \sim 1 \times 10^6 \) cells/ml using culture media containing 10% dimethyl sulfoxide (DMSO) and 40% FCS. Cells were stored in liquid nitrogen until use.

2.3 Purification of plasma-derived HRG

Native human HRG was purified from fresh human plasma as previously described (Rylatt et al., 1981). Briefly, a phosphocellulose column was prepared by adding a mixture of 500 ml of 0.2 M HCl and 500 ml of ethanol to 35 g of Whatman P-11 phosphocellulose (Whatman, Kent, United Kingdom) and stirred gently using a glass rod for 30 mins. The phosphocellulose was then washed sequentially with 1 l of DDW, 1 l of 0.1 M NaOH, 1 l of DDW and resuspend in 1 l of 1 mM EDTA for 30 mins with occasional stirring. The phosphocellulose was washed again with 1 l of DDW and resuspend in 500 ml of 0.5 M NaCl loading buffer (10 mM NaP, 0.5 M NaCl, 1 mM EDTA, pH 6.8). All subsequent purification steps were performed at 4°C to avoid degradation of proteins. The phosphocellulose was then poured into a 20 cm BioRad column (BioRad, Richmond, CA), washed with 90 ml of 0.5 M NaCl loading buffer containing 50 mg/ml of BSA, followed by 90 ml of 0.5 M NaCl loading buffer, 90 ml of 2 M NaCl loading buffer, before final equilibration with 0.5 M NaCl loading buffer. Approximately 500 ml of fresh human plasma (Red Cross, The Canberra Hospital, Canberra, Australia), in the presence of 1 mM EDTA, 0.5 M NaCl, 100 \( \mu \)g/ml Pefabloc (Roche, Indianapolis, IN) and 2 \( \mu \)g/ml aprotinin (Boehringer, Mannheim, Germany), was centrifuged at 10000 g for 20 min in a Sorvall RC-5B Refrigerated Superspeed Centrifuge (Dupont, Wilmington, DE). The plasma supernatant was then passed through the equilibrated phosphocellulose column and unbound proteins were removed from the column with 1 l of 0.5 M NaCl loading buffer and subsequently 300 ml of 0.7 M NaCl loading buffer. Column-bound HRG was finally eluted with 2 M NaCl loading buffer, concentrated and buffer exchanged against buffer containing 10 mM NaP and 0.5 M NaCl using a Diaflo Concentrator 202 and YM-30 Diaflo Ultrafiltration Membrane.
Chapter 2

(Purified Plasma-derived HRG (HRG\textsuperscript{P}) was aliquoted and stored at -70°C until use.

2.4 Purification of endotoxin-free HRG\textsuperscript{P}

EndoTrap\textsuperscript{®} red column (Profos AG, Regensburg, Germany) was used to remove traces of endotoxin/lipopolysaccharide (LPS) present in HRG\textsuperscript{P} according to the manufacturers' instructions, in which all reagents required were provided by the manufacturer. Briefly, EndoTrap\textsuperscript{®} red column (containing 1 ml of EndoTrap\textsuperscript{®} red resin) was washed twice with 3 ml of regeneration buffer, twice with 3 ml of equilibration buffer and then loaded with 200 µl of HRG\textsuperscript{P} (5 mg/ml). The column was washed with 2 ml of equilibration buffer and the flow-through (containing endotoxin-free HRG\textsuperscript{P}) was collected into ~100 µl fractions. All fractions were tested for the presence of protein via the Bradford protein assay as described in Section 2.9 and protein-containing fractions were pooled appropriately. Purified endotoxin-free HRG\textsuperscript{P} was aliquoted and stored at -70°C until use.

2.5 Protein G column purification of human and mouse IgG

HiTrap\textsuperscript{TM} Protein G column (Amersham Biosciences, Buckinghamshire, United Kingdom) was used to affinity purify human or mouse IgG. Briefly, HiTrap\textsuperscript{TM} Protein G column (containing 1 ml of Protein G Sepharose\textsuperscript{TM} High Performance) was washed with 10 ml of PBS and loaded with 4 to 8 ml of sample slowly using a 5 ml syringe. The column was then washed with 5 ml of PBS and the flow-through (containing sample constituents that did not bind to Protein G) was collected into ~1 ml fractions. Column-bound IgG was eluted using 5 ml of elution buffer (0.1M glycine-HCl, pH 2.7) and collected into ~1 ml fractions. All fractions were tested for the presence of protein via the Bradford protein assay (see Section 2.9) and protein-containing fractions were pooled appropriately and concentrated using a Centricon YM-30 or Centricon YM-100 concentrator (Millipore, Milford, MA). Purified proteins including Plasma-Derived IgG-Depleted HRG (HRG\textsuperscript{PID}), the co-purified IgG isolated from HRG\textsuperscript{P} (IgG\textsuperscript{HRG}) and a
mouse anti-human HRG monoclonal antibody (mAb) (HRG-4) were aliquoted and stored at -70°C until use.

### 2.6 Biotinylation of purified proteins

Purified proteins were excessively biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Pierce Biotechnology, Rockford, IL) to detect for contaminating proteins in the preparation. 10 μl of EZ-Link Sulfo-NHS-LC-Biotin (11.11 mg/ml) was added to 10 μl of purified proteins (500 μg/ml) in PBS and incubated at 4°C for 20 hrs. Biotinylated proteins were stored at -20°C until use.

HRG<sup>PID</sup> was biotinylated using EZ-link Maleimide PEO<sub>2</sub>-biotin (Pierce Biotechnology, Rockford, IL) for flow cytometry-based cell binding studies. Briefly, 10 μl of EZ-link Maleimide PEO<sub>2</sub>-biotin (20 mg/ml) was added to 600 μl of HRG<sup>PID</sup> (1 mg/ml) in PBS and incubated at 4°C for 4 hrs. To remove excess non-reacted and hydrolyzed biotin reagent, biotinylated HRG<sup>PID</sup> was concentrated and washed with 6 ml of PBS using a Centricon YM-30 concentrator. Biotinylated proteins were stored at -20°C until use.

### 2.7 Generation of plasmin-cleaved HRG

Plasmin-cleaved HRG used in Chapter 4 and 5 of this thesis was generated via the same procedure with minor modifications depending on the application. For the initial analysis of the proteolytic cleavage of HRG by plasmin and the preparation of samples for Edman N-terminal sequencing, plasmin-mediated digestion of HRG was perform at 37°C using 1 mg/ml of HRG<sup>PID</sup> and 50 μg/ml of plasmin (Sigma-Aldrich, St. Louis, MO) in PBS (pH 7.2). For ELISA- and flow cytometry-based binding assays and Western Blot analysis using a mouse anti-human HRG mAb (HRG-4), proteolytic cleavage of HRG by was performed at 37°C using 300 μg/ml of HRG<sup>PID</sup> and 30 μg/ml of plasmin in PBS (pH 7.2). Following plasmin treatment, HRG<sup>PID</sup> preparations were diluted in appropriate buffer containing 50 μg/ml of aprotinin (Boehringer, Mannheim, Germany) to inhibit further digestion of HRG by plasmin during the subsequent ELISA-
and flow cytometry-based binding assays. To investigate the effect of heparin (12.5 kDa), pH and Zn\(^{2+}\) on the proteolytic cleavage of HRG by plasmin, 30 µg/ml of HRG\(^{PID}\) and 6 µg/ml of plasmin were diluted in PBS and incubated at 37°C for 60 mins under the different conditions.

### 2.8 Generation of F(ab')\(_2\) fragments

ImmunoPure® IgG1 Fab and F(ab')\(_2\) preparation kit (Pierce Biotechnology, Rockford, IL) was used to generate a mouse F(ab')\(_2\) anti-human FcγRI mAb (10.1) and a mouse F(ab')\(_2\) anti-human HRG mAb (HRG-4) according to the manufacturers' instructions, in which all reagents required were provided in the kit. Briefly, 2 ml column of immobilized ficin was washed with 15 ml of working digestion buffer. 100 µl of stock digestion buffer containing 2 mg/ml of cysteine.HCl was added to 1 ml of mouse mAb (400 µg/ml) and the sample was applied to the immobilized ficin column. To generate F(ab')\(_2\) fragments, the immobilized ficin column was incubated at 37°C for 20 hrs and a 4 ml fraction of the digested sample was collected by adding 4 ml of ImmunoPure® binding buffer to the column. To separate F(ab')\(_2\) fragments from Fc fragments and non-digested IgG, the digested sample was passed through an AffinityPak™ Immobilized Protein A column and the flow-through (containing only the F(ab')\(_2\) fragments) was collected, pooled appropriately based on protein concentration and concentrated using a Centricon YM-30 concentrator. Mouse F(ab')\(_2\) anti-human FcγRI mAb (10.1) and mouse F(ab')\(_2\) anti-human HRG mAb (HRG-4) were stored at 4°C or -20°C, respectively, until use. Purity of F(ab')\(_2\) mAbs were confirmed by Western blot analysis (see Section 2.13, data not shown).

### 2.9 Bradford protein assay

The Bradford protein assay was used to estimate protein concentration. Briefly, BSA standards and protein samples were diluted in PBS to a total volume of 100 µl and added to a U-bottomed 96 well PVC microtitre plate (Dynex Technologies, Chantilly, VA). The Bradford reagent concentrate dye (BioRad, Hercules, CA) was diluted in the ratio of
1:4 with DDW and 100 µl was then added to each standard and sample. The optical
density was measured at 595 nm using a Thermomax microplate reader. Data were
analyzed by SoftMaxPro 4.0 software (Molecular Devices, Sunnyvale, CA).

2.10 Enzyme-linked immunoabsorbent assay (ELISA)

2.10.1 Standard ELISA for measuring protein-protein interactions

ELISAs were performed by coating U-bottomed 96 well PVC microtitre plates with the
protein to be immobilized (1-10 µg/ml, 50 µl/well) in PBS for 16 hrs at 4°C. Unbound
protein was removed by gently flicking the plate and wells were then washed 3 times
(100 µl/well) with PBS/0.05% Tween-20. ELISA wells were then incubated with
PBS/3% BSA (200 µl/well) for 3 hrs at 4°C to block non-specific binding. Binding
proteins diluted in PBS/1% BSA were then added (50 µl/well) and the plates incubated
for 90 mins at 4°C. Wells were washed 3 times (100 µl/well) with PBS/0.05% Tween-
20, protein specific Ab diluted in PBS/1% BSA added (50 µl/well) and the plates
incubated for 90 mins at 4°C. Wells were then washed 3 times (100 µl/well) with
PBS/0.05% Tween-20 and horseradish peroxidase (HRP)-conjugated secondary Ab
diluted in PBS/1% BSA was added (50 µl/well) for 90 mins at 4°C. Wells were washed
3 times and well-bound HRP-conjugated Ab was detected using 100 µl/well of the
peroxidase substrate, 2,2'-azido-bis(3-ethylbenzthiazol-6-sulphonic acid) (ABTS)
(Kirkegaard and Perry Laboratories, Gaithersburg, MD). The absorbance of the
enzymatic product at 405 nm was measured using a Thermomax microplate reader. Data
were analyzed by SoftMaxPro 4.0 software. Primary and secondary Abs used in
different ELISAs are listed in Table 2.3.

2.10.2 ELISA for detecting lipid-protein interactions

The ELISA protocol used was as described in Section 2.10.1, with the exception that
when being coated on microtitre plates, phospholipids were diluted in ethanol
Table 2.3. ELISA protocols for specific primary and secondary antibodies.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Primary antibody (clone) / dilution / supplier</th>
<th>Secondary antibody / dilution / supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human HRG</td>
<td>Mouse anti-human HRG (HRG-4) / 1:1000 / AGEN, Brisbane, Australia</td>
<td>Sheep anti-mouse Ig-HRP / 1:1000 / Chemicon Australia, Melbourne, Australia</td>
</tr>
<tr>
<td>Human Ig γ H-chain</td>
<td>Rabbit anti-human IgG-HRP, γ H-chain specific / 1:500 / Dako, Glostrup, Denmark</td>
<td></td>
</tr>
<tr>
<td>Human plasminogen</td>
<td>Rabbit anti-human plasminogen / 1:500 / Dako, Glostrup, Denmark</td>
<td>Sheep anti-rabbit Ig-HRP / 1:1000 / Chemicon Australia, Melbourne, Australia</td>
</tr>
</tbody>
</table>

HRP, Horseradish peroxidase; Ig, immunoglobulin

(10-50 μg/ml, 50 μl/well) and incubated at RT for 16 hrs to allow evaporation of the ethanol prior to blocking with PBS/3% BSA. In addition, all wash steps were performed using PBS without the detergent Tween-20.

2.10.3 ELISA for human IgG subclass profiling

Human IgG subclass profile ELISA kit (Zymed® Laboratories, San Francisco, CA) was used to estimate the amount of different human IgG subclasses present in pooled normal human IgG and IgG²HRG (the co-purified IgG isolated from HRG³) according to the manufacturers’ instructions, in which all reagents required were provided in the kit. Briefly, microtiter plates that can capture monoclonal reagents were washed once (200 μl/well) with wash buffer and a mixture contain 50 μl of read-to-use standard or sample and 50 μl of the appropriate human IgG subclass specific mAb were added to each well for 30 mins at RT. Wells were then washed 3 times (200 μl/well) with wash buffer and 100 μl of HRP-conjugated anti-human IgG Ab was added to each well for 30 mins at RT. Wells were washed three times (200 μl/well) with wash buffer and well-bound HRP-conjugated Ab was detected using 100 μl/well of the ready-to-use 3.3',5.5'-tetramethylbenzidine (TMB) substrate. Wells were then incubated for 10 mins at RT and the enzymatic reaction was stopped by adding 50 μl/well of stopping reagent. The absorbance of the enzymatic product was measured at 450 nm on a Thermomax microplate reader. Data were analyzed by SoftMaxPro 4.0 software.
2.11 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were mixed with an equal volume of 2x reducing or non-reducing sodium dodecyl sulfate (SDS) sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS), with the reducing buffer containing 10% dithiothreitol (DTT, reducing agent), and boiled for 5 mins. Samples were then subjected to electrophoresis (80 volts, 60-120 mins) on a 4-20% gradient or 10% precast polyacrylamide MiniGel (Gradipore, Sydney, Australia) using a Mini-Protean II apparatus (BioRad, Hercules, CA) in SDS-HEPES running buffers (Gradipore, Sydney, Australia). 5 µl of prestained molecular weight standards (BioRad, Hercules, CA) were run simultaneously to estimate the apparent molecular weight of protein samples.

2.12 Coomassie Brilliant Blue protein staining

Following SDS-PAGE, protein samples were stained with 0.2% Coomassie Brilliant Blue (0.2% Coomassie Brilliant Blue, 50% methanol, 7.5% acetic acid) for 16 hrs at RT and then destained with destain buffer (40% methanol, 7% acetic acid) at RT until discrete protein bands could be visualized clearly.

2.13 Western blotting

Following SDS-PAGE, protein samples were transferred electrophoretically (80 volts, 120 mins) from the polyacrylamide gel onto a nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, United Kingdom) using a Mini-Protean II apparatus in transfer buffer containing 25 mM Tris, 192 mM glycine and 20% methanol. The membrane was incubated with PBS/5% skim milk powder (SMP) for 16 hrs at 4°C to block non-specific binding. The membrane was then incubated with protein specific Ab diluted in PBS/0.05% Tween-20/5% SMP or PBS/1% BSA for 90 mins at 4°C and washed extensively for 60 mins at RT with PBS/0.05% Tween-20. The membrane was incubated with HRP-conjugated secondary Ab diluted in PBS/0.05% Tween-20/5% SMP or PBS/1% BSA for 90 mins at 4°C and washed thoroughly for 60 mins at RT with
Chemiluminescence was detected using the enhanced chemiluminescence (ECL) Western blotting reagent (Amersham Biosciences, Buckinghamshire, United Kingdom) and imaged using the FujiFilm Image Reader Las1000 Lie V1.5 (Fujifilm, Tokyo, Japan). Data were analyzed by Image Gauge V3.46 software (Fujifilm, Tokyo, Japan). Primary and secondary Abs used in different Western blots are listed in Table 2.4.

Table 2.4. Western blotting protocols for specific primary and secondary antibodies.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Primary antibody (clone) / dilution / supplier</th>
<th>Secondary antibody / dilution / supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human HRG</td>
<td>Mouse anti-human HRG (HRG-3 or HRG-4) / 1:1000 / AGEN, Brisbane, Australia or Rabbit anti-human HRG (115, 116 or 119) / 1:3000 / Gift from Dr. Anna-Karin Olsson, Sweden</td>
<td>Sheep anti-mouse Ig-HRP / 1:1000 / Chemicon Australia, Melbourne, Australia or Sheep anti-rabbit Ig-HRP / 1:1000 / Chemicon Australia, Melbourne, Australia</td>
</tr>
<tr>
<td>Human Ig γ H-chain</td>
<td>Rabbit anti-human IgG-HRP, γ H-chain specific / 1:500 / Dako, Glostrup, Denmark</td>
<td></td>
</tr>
<tr>
<td>Human Ig κ L-chain</td>
<td>Mouse anti-human Ig, κ L-chain specific (KP-53) / 1:500 / Sigma, St. Louis, MO</td>
<td></td>
</tr>
<tr>
<td>Human Ig λ L-chain</td>
<td>Mouse anti-human Ig, λ L-chain (HP-6054) / 1:500 / Sigma, St. Louis, MO</td>
<td></td>
</tr>
<tr>
<td>Human C1q</td>
<td>Mouse anti-human C1q (MhC5B9) / 1:500 / Cedarlane, Ontario, Canada</td>
<td></td>
</tr>
<tr>
<td>Human heparanase</td>
<td>Rabbit anti-human heparanase / 1:2000 / InSight Biopharmaceuticals, Israel</td>
<td></td>
</tr>
<tr>
<td>Biotinylated proteins</td>
<td>Streptavidin-HRP / 1:1000 / Amersham Biosciences, Buckinghamshire, United Kingdom</td>
<td></td>
</tr>
<tr>
<td>Histidine residues</td>
<td>NINTA-HRP / 1:1000 / KPL, Gaithersburg, MD</td>
<td></td>
</tr>
</tbody>
</table>

HRP, horseradish peroxidase; Ig, immunoglobulin; NTA, nitrilotriacetic acid

2.14 Lipid-coated membrane strip-based binding assay

Membrane Lipid Strips™, PIP Strips™ and SphingoStrips™ (Echelon Biosciences, Salt Lake City, UT) were incubated with PBS/3% fatty acid-free BSA for 16 hrs at RT to block non-specific binding. The membrane strips were then incubated with HRG PID (1 μg/ml) diluted in PBS/3% fatty acid-free BSA for 90 mins at 4°C and washed
thoroughly for 60 mins at RT with PBS/0.1% Tween-20. Membrane-bound HRG was detected by probing the membrane strips with a mouse anti-human HRG mAb (HRG-4) diluted in PBS/3% fatty acid-free BSA for 90 mins at 4°C, followed by a HRP-conjugated sheep anti-mouse Ig Ab diluted in PBS/3% fatty acid-free BSA for 90 mins at 4°C. After each antibody incubation the membrane strips were washed extensively for 60 mins at RT with PBS/0.1% Tween-20. Chemiluminescence was detected as described in Section 2.13.

2.15 Densitometry analysis of Western blot and lipid-coated membrane strip

Densitometry analysis was performed on images obtained from Western blot and lipid membrane strip using the Image Gauge V3.46 software. Briefly, boxes/circles of equivalent size were traced around bands/areas of interest. A background box/circle of equal size was also placed in an area on the membrane where no specific chemiluminescence could be detected and set as ‘global’ background. The intensity of bands/areas of interest was quantified as the pixel intensity per mm$^2$ subtracting the ‘global’ background.

2.16 Cytoplasmic protein pull-down assay

2.16.1 Generation of cytoplasmic lysate

Human MM170 cells were washed twice with PBS and ~1 x 10$^6$ cells were lysed by resuspending cells in 200 µl of PBS containing 0.5% Triton X-100 and 1x cocktail protease inhibitors (Roche Diagnostics, Mannheim, Germany) for 30 mins at 4°C. To pellet membrane and nuclear materials, lysed cells were spun at 13000 rpm for 15 mins at 4°C and the supernatant containing cytoplasmic lysate was stored at -70°C until use.
2.16.2 Generation of HRG\textsuperscript{PID}-conjugated Sepharose beads

HRG\textsuperscript{PID} (1.5 mg) was incubated with a 10-fold excess of acetylated heparin (2.5 mg) in 2 ml of PBS for 30 mins at 4°C to block the heparin-binding sites on HRG\textsuperscript{PID} during the conjugation process. 1.5 ml of NHS-activated Sepharose® beads (Amersham Pharmacia Biotech, Amersham, Sweden) were washed once with 5 ml of 1 mM HCl, 3 times with PBS and then incubated with the 2 ml mixture containing HRG\textsuperscript{PID} and acetylated heparin for 16 hrs at 4°C. Non-reacted N-hydroxysuccinimide (NHS) was inactivated by incubating HRG\textsuperscript{PID}-beads in 2 ml of 1 M Tris (pH 7.9) for 3 hrs at 4°C and then washed twice with buffer containing 2 M NaCl and 100 mM Tris to remove acetylated heparin. HRG\textsuperscript{PID}-beads were washed 3 times with PBS and stored at 4°C until use.

2.16.3 Pull-down assay

HRG\textsuperscript{PID}- and BSA-conjugated Sepharose beads (30 µl) were washed once with 2 M NaCl and twice with PBS prior to incubation with 70 µl of isolated cytoplasmic lysate for 1 hr at 4°C. HRG\textsuperscript{PID}- and BSA-beads were washed 3 times with PBS and beads-bound proteins were analyzed by SDS-PAGE and Coomassie Brilliant Blue staining as described in Section 2.11 and 2.12.

2.17 Cell killing protocols

Jurkat T cells were induced to become apoptotic or necrotic/permeabilized cells via several methods. To induce apoptotic and secondary necrotic cells, cells were cultured in serum-free RPMI-1640 for 48 hrs. To induce primary necrotic cells, cells were exposed to hyperthermic conditions (56°C) for 30 mins. To produce permeabilized cells, cells were either exposed to one freeze/thaw cycle or treated with 0.05% Triton X-100 for 15 mins at 4°C. Dying/dead cells were washed twice with PBS and resuspended in appropriate buffer depending on the subsequent application.
2.18 Treatment of viable or necrotic cells with various enzymes

2.18.1 Treatment of viable THP-1 cells or necrotic Jurkat T cells with heparanase

To enzymatically remove heparan sulfate (HS) exposed on viable THP-1 cells or necrotic Jurkat T cells, cells were treated with 4 µg/ml of human platelet-derived heparanase for 60 mins at 37°C in serum-free RPMI-1640/0.1% BSA.

2.18.2 Treatment of necrotic Jurkat T cells with DNase I

To enzymatically remove DNA exposed in necrotic Jurkat T cells, cells were treated with 100 or 1000 µg/ml of bovine DNase I (Roche Diagnostics, Mannheim, Germany) for 90 mins at 37°C in buffer containing 0.15 M NaCl and 4.2 mM MgCl₂.

2.18.3 Treatment of necrotic Jurkat T cells with phospholipase C

To enzymatically remove the phosphate headgroup of various phospholipids exposed in necrotic Jurkat T cells, cells were treated with 2 unit/ml of Bacillus cereus phospholipase C (Sigma-Aldrich, St. Louis, MO) in PBS for 90 mins at 37°C.

2.18.4 Treatment of necrotic Jurkat T cells with alkaline phosphatase

To enzymatically dephosphorylate various proteins on necrotic Jurkat T cells, cells were treated with 250 unit/ml of calf alkaline phosphatase (Promega, Madison, WI) for 60 mins at 37°C in buffer containing 50 mM Tris-HCl, 1 mM MgCl₂, 0.1 mM ZnCl₂ and 1 mM spermidine.

2.19 Immunofluorescence flow cytometry

Immunofluorescence flow cytometry was used to analyze protein binding to cells, as well as detecting the expression/exposure of various molecules. Table 2.5 outlines
Table 2.5. Immunofluorescence protocols for specific primary and secondary antibodies.

<table>
<thead>
<tr>
<th>Specificity</th>
<th>Primary antibody (clone) / dilution / supplier</th>
<th>Secondary antibody / dilution / supplier</th>
</tr>
</thead>
</table>
| Human HRG  | Mouse anti-human HRG (HRG-4) / 1:200 / AGEN, Brisbane, Australia  
Mouse F(ab')₂, anti-human HRG (HRG-4) / 1:200 / AGEN, Brisbane, Australia | Sheep F(ab')₂ anti-mouse Ig-FITC / 1:50 / Amrad Biotech, Melbourne, Australia  
or Sheep F(ab')₂ anti-mouse Ig-PE / 1:100 / Chemicon Australia, Melbourne, Australia |
| Human Igγ H-chain | Goat anti-human IgG-PE, γ H-chain specific / 1:500 / Sigma, St. Louis, MO | Sheep F(ab')₂ anti-mouse Ig-PE / 1:100 / Chemicon Australia, Melbourne, Australia |
| Human C1q | Mouse anti-human C1q (MhC5B9) / 1:100 / Cedarlane, Ontario, Canada | Sheep F(ab')₂ anti-mouse Ig-PE / 1:100 / Chemicon Australia, Melbourne, Australia |
| Human FcyRI | Mouse F(ab')₂ anti-human FcyRI (10.1) / 1:100 / eBioscience, San Diego, CA | Sheep F(ab')₂ anti-mouse Ig-PE / 1:100 / Chemicon Australia, Melbourne, Australia |
| Human FcyRIIA | Mouse F(ab')₂, anti-human FcyRIIA (8.26) / 1:30 / (Ierino et al., 1993) | Sheep F(ab')₂ anti-mouse Ig-PE / 1:100 / Chemicon Australia, Melbourne, Australia |
| Heparan sulfate | Mouse anti-heparan sulfate (F58-10E4) / 1:100 / Seikagaku, Tokyo, Japan | Sheep F(ab')₂ anti-mouse Ig-PE / 1:100 / Chemicon Australia, Melbourne, Australia |
| Human CIMPR | Mouse anti-CIMPR (MEM-328) / 1:100 / Abcam, Cambridge, United Kingdom | Sheep F(ab')₂ anti-mouse Ig-PE / 1:100 / Chemicon Australia, Melbourne, Australia |
| Biotinylated proteins | Streptavidin-PE / 1:500 / BD Pharminogen, San Diego, CA  
or Streptavidin-APC / 1:800 / BD Pharminogen, San Diego, CA | |
| Phosphatidylserine | Annexin V-FITC / 1:50 / BD Pharminogen, San Diego, CA | |
| Phosphoserine/threonine | Mouse anti-phosphoserine/threonine (22A) / 1:50 / BD Pharminogen, San Diego, CA | Sheep F(ab')₂ anti-mouse Ig-PE / 1:100 / Chemicon Australia, Melbourne, Australia |

APC, allophycocyanin; CIMPR, cation-independent mannose-6-phosphate receptor; FcyR, Fcγ receptor; FITC, fluorescein isothiocyanate; Ig, immunoglobulin; PE, R-phycoerythrin

Different staining protocols used in immunofluorescence flow cytometry. Typically, ~2 x 10^5 cells were resuspended in PBS/0.1% BSA (pH 7.2) and added to a V-bottomed 96 well plate. Binding proteins (e.g. HRG) diluted in PBS/0.1% BSA (pH 7.2) were then added (10-20 μl/well) and incubated for 30 mins at 4°C. Cells were then washed 3 times with PBS/0.1% BSA (pH 7.2) and protein binding was detected by incubating cells with appropriate primary Ab diluted in PBS/0.1% BSA (pH 7.2) for 30 mins at 4°C. Cells were subsequently washed 3 times with PBS/0.1% BSA (pH 7.2) and incubated with appropriate fluorescence-conjugated secondary Ab diluted in PBS/0.1% BSA (pH 7.2).
for 30 mins at 4°C. Finally, cells were washed 3 times with PBS/0.1% BSA (pH 7.2), resuspended in PBS/0.1% BSA (pH 7.2) containing 1 µg/ml of Hoechst 33258 (Calbiochem, La Jolla, CA) and analyzed by flow cytometry using a LSR1 Flow Cytometer and Cell Quest Pro software (BD Biosciences, San Jose, CA). The resultant flow cytometry data were analyzed by FlowJo software (Tree Star, Ashland, OR). Samples that were incubated with primary Ab and fluorescence-conjugated secondary Ab in the absence of binding proteins were used as negative controls to set up appropriate laser voltage. Single colour positive controls were used to set up appropriate compensation parameters. Cells were gated appropriately based on forward scatter (FSC) and side scatter (SSC). Live and dead cells were distinguished based on Hoechst 33258 negative and positive staining, respectively.

2.20 Phagocytic assay

The human monocytic cell line THP-1 was used as the phagocytic cell and labeled with PKH26 (Sigma-Aldrich, St. Louis, MO). Briefly, ~2 x 10^6 cells were washed once with PBS and resuspended in 150 µl of PBS. Pre-diluted PKH26 containing 10 µl of Diluent C and 2 µl of PKH26 was added to the cell suspension for 5 mins at RT. To stop the staining reaction, 150 µl of 10% BSA was added for 1 min at RT. Cells were then washed 4 times with serum-free RPMI-1640 and resuspended to a cell concentration of ~1 x 10^6 cells/ml with serum-free RPMI-1640/0.1% BSA.

Jurkat T cells were labeled with carboxy-fluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes, Leiden, The Netherlands) and induced into necrosis by exposure to hyperthermic conditions. Briefly, cells were resuspended to a cell concentration of ~2 x 10^6 cells/ml in serum-free RPMI-1640 containing 1 µM CFSE and incubated for 15 mins at 37°C. Cells were washed 4 times with serum-free RPMI-1640 and induced into necrosis by incubating the cells at 56°C for 30 mins. Cells were then washed once with serum-free RPMI-1640 and resuspended to a cell concentration of ~1 x 10^7 cells/ml with serum-free RPMI-1640/0.1% BSA.
The phagocytic assay was performed immediately by incubating 15 μl/well of PKH26-labeled THP-1 cells (~1 x 10^6 cells/ml) with 15 μl/well of CFSE-labeled necrotic Jurkat T cells (~1 x 10^7 cells/ml) at a cell ratio of 1:10 in a pre-warmed U-bottomed 96 well plate. Samples were then incubated for 60 mins at 37°C in a humidified atmosphere containing 5% CO₂. Samples were immediately placed on ice and analyzed by flow cytometry using a LSR1 Flow Cytometer and Cell Quest Pro software. Percentage of phagocytosis was determined as the percentage of PKH26 positive phagocytic cells that had ingested CFSE positive necrotic Jurkat T cells (see Fig. 2.1A and B below).

![Flow cytometry analysis of the phagocytosis of necrotic Jurkat T cells by the human monocytic cell line THP-1.](image)

**Figure 2.1.** Flow cytometry analysis of the phagocytosis of necrotic Jurkat T cells by the human monocytic cell line THP-1. (A) Representative flow cytometry plot is shown for phagocytic assay performed at 37°C for 60 mins in the absence of serum opsonins, in which ~5 to 15% of PKH26 positive THP-1 cells were able to bind and ingest CFSE positive necrotic Jurkat T cells. (B) Mathematical equation used to determine the percentage of phagocytosis.

\[
\text{Percentage of phagocytosis} = \frac{\text{Percentage of CFSE positive THP-1 cells (top right gate)}}{\text{Total percentage of THP-1 cells (top left and right gate)}} \times 100
\]

2.21 Human inflammation cytokine Cytometric Bead Array (CBA) assay

Cytokine secretion by THP-1 cells was measured using a BD™ CBA human inflammation kit (BD Biosciences, San Jose, CA) according to the manufacturers' instructions, in which all reagents required were provided in the kit. Cytokines examined were human IL-8, IL-1β, IL-6, IL-10, TNF and IL-12p70. Briefly, a lyophilized
cytokine standard was reconstituted in 45 μl of DDW to make the top standard containing 5000 pg/ml of each cytokine and 1:2 serial dilutions were made to a concentration of 5 pg/ml. The capture beads for each cytokine (2.5 μl/sample) were combined and 12.5 μl of the mixed capture beads was added to a V-bottomed 96 well plate for each sample and standard. 12.5 μl of human inflammation PE-detection reagent and 12.5 μl of sample or standard were also added to each well and incubated for 3 hrs at RT. Capture beads were washed once with 200 μl/well of wash buffer and centrifuged at 200 g for 5 mins. The supernatant was discarded and the capture beads were resuspended in 100 μl/well of wash buffer and analyzed by flow cytometry using a LSR1 Flow Cytometer and Cell Quest Pro software. The resultant flow cytometry data were analyzed using FCAP Array Software (Soft Flow for BD Biosciences, San Jose, CA).

### 2.22 Confocal laser scanning microscopy (CLSM) imaging

Stained cells were mounted onto glass microscope slides by placing a drop of cell suspension on the slide and laying a coverslip onto the cell suspension. Slides were sealed using nail polish and stored at 4°C prior to imaging. Cells were imaged with CLSM using a Nikon Eclipse TE 300 confocal microscope with Nikon Super High Pressure Mercury Lamp power supply (Nikon, Tokyo, Japan) and a Radiance 2000 Laser Scanning System (BioRad, Hercules, CA). Images were captured with variable gain level and Kalman filter mode (3 scans), with the resultant data being analyzed by Laser Sharp 2000 software (BioRad, Hercules, CA).

### 2.23 Plasmin activity assay

The chromogenic plasmin substrate N-(p-Tosyl)-Gly-Pro-Lys (Sigma-Aldrich, St. Louis, MO) was used to assess plasmin activity. Briefly, samples were prepared in 50 μl of PBS (pH 7.2, 6.6 or 6.0) containing 1 mg/ml of N-(p-Tosyl)-Gly-Pro-Lys and incubated for 60 mins at 37°C. Samples were then transferred into a U-bottomed 96 well PVC
microtitre plate and the absorbance of the enzymatic product was measured at 405 nm on a Thermomax microplate reader. Data were analyzed by SoftMaxPro 4.0 software.


CPC is a cationic compound that binds to HS of different sizes and precipitates out of solution according to the salt concentration. A salt concentration of 140 mM was chosen to precipitate out the larger/intact $[^3]$H-HS fragments, leaving the smaller/heparanase-cleaved $[^3]$H-HS fragments in solution to be measured by the scintillation counter (Packard, Downers Grove, IL). Briefly, a final concentration of 0.2 µg/ml of human platelet-derived heparanase was diluted in 40 µl of substrate cocktail containing $[^3]$H-HS (50 µg/ml), BSA (10 µg/ml), and NaOAc (40 µM, pH 5.1 or 7.2) in DDW. Samples were incubated at 37°C for 30 mins at pH 5.1 or 60 mins at pH 7.2 to achieve suboptimal cleavage of $[^3]$H-HS by heparanase at specific pH. Samples were then frozen immediately on dry ice to stop further cleavage of $[^3]$H-HS by heparanase. Samples were then thawed at RT and 40 µl of unlabeled heparin (10 mg/ml) was added to inhibit further cleavage of $[^3]$H-HS by heparanase and function as a vehicle for the precipitation process. To precipitate the larger/intact $[^3]$H-HS fragments, samples were incubated with 340 µl of CPC solution (1% CPC, 175 mM NaCl) for 30 mins at 37°C. To determine the radioactivity in the soluble solution containing the smaller/heparanase-cleaved $[^3]$H-HS fragments, the precipitate was pelleted by centrifugation at 3000 rpm for 15 mins and 100 µl of the supernatant was transferred to a scintillation vial containing 200 µl of DDW. For each sample, 3.6 ml of scintillation fluid was added and the radioactivity was measured by the scintillation counter.

2.25 Statistical analysis

Statistical significance was measured using students t-test (unpaired, two-tailed) performed by InStat 2.01 software (GraphPad Software, San Diego, CA).
HISTIDINE-RICH GLYCOPROTEIN COMPLEXES WITH IgG TO FACILITATE THE PHAGOCYTOSIS OF NECROTIC CELLS

3.1 Abstract

Under normal physiological conditions, necrotic cells resulting from tissue injury are rapidly removed from the circulation and tissues by phagocytic cells, thus preventing the exposure of intracellular antigenic and immunostimulatory molecules that can aid the development of autoimmune disease. Histidine-rich glycoprotein (HRG), a relatively abundant (~100-150 µg/ml) plasma glycoprotein, has a multi-domain structure that can interact with many ligands including heparin, heparan sulfate (HS), Fcγ receptors (FcγR), IgG and C1q. Recently, studies have reported that HRG can bind strongly to cytoplasmic ligand(s) exposed in necrotic cells and enhances necrotic cell clearance by phagocytes. To elucidate the molecular mechanisms underpinning this process, experiments described in Chapter 3 demonstrate that a complex consisting of both HRG and IgG is necessary to aid necrotic cell uptake by the monocytes cell line THP-1, predominantly via a FcγRI- and HS-dependent mechanism. Interestingly, the findings in
this chapter also show that HRG can potentially interact with anionic phospholipids and phosphorylated proteins exposed in necrotic cells. Furthermore, the enhanced phagocytosis of necrotic cells induced by HRG-IgG complexes triggers phagocytes to release pro-inflammatory cytokines such as IL-8 and TNF. Thus, HRG has the unique property of complexing with IgG and facilitating a pro-inflammatory innate immune response to promote the clearance of necrotic cells, as well as attracting leukocytes to sites of tissue injury.

3.2 Introduction

The immune system plays a vital role in accurately detecting and distinguishing foreign pathogens/materials from normal healthy tissues in order to prevent the invasion of foreign substances. It is equally important for the immune system to discriminate healthy viable cells from dying/dead cells during the course of development, tissue remodelling and tissue injury to avoid the release of intracellular molecules from dying/dead cells that may damage neighbouring cells. Professional phagocytes of the innate immune system utilize a broad range of germ-line encoded receptors and opsonins to specifically detect pathogens/dying/dead cells and aid their removal via the process of phagocytosis (Gregory and Devitt, 2004; Stuart and Ezekowitz, 2005).

When viable cells undergo apoptosis in response to either extrinsic or intrinsic mediators, a precise set of morphological (e.g. cell shrinkage and blebbing) and biochemical (e.g. activation of caspases) changes occur (Fink and Cookson, 2005). To achieve the efficient clearance of dying cells, apoptotic cells release 'come-get-me' signals (e.g. lysophosphatidylcholine) to recruit phagocytes to the site of cell death and expose a specific combination of 'eat-me' and 'don’t eat-me' signals to trigger phagocytosis (Grimsley and Ravichandran, 2003). Typically, the loss of phospholipid asymmetry by the plasma membrane during the early stages of apoptosis can lead to the exposure of phosphatidylyserine (PS), which can function as an 'eat-me' signal to aid the uptake of apoptotic cells via a diverse range of phagocytic receptors and opsonins such as the PS receptor (PSR) and scavenger receptors, as well as the opsonins β2-
glycoprotein I (β2GPI) and milk fat globule-EGF factor 8 protein (MFG-E8) (Grimsley and Ravichandran, 2003). However, when apoptotic cells persist due to an overload of dying cells and/or an impairment in phagocytosis, early apoptotic cells will progress into late apoptotic and secondary necrotic cells whereby the cell membrane becomes more permeable (Munoz et al., 2005). Similarly, generation of primary necrotic cells by extreme trauma can also result in the permeabilization of the cell membrane (Zhivotovsky and Orrenius, 2001). The loss of membrane integrity exposes intracellular contents, which may function as additional ‘eat-me’ signals to aid the removal of permeabilized/necrotic cells (Krysko et al., 2006). Most importantly, unlike the recognition of pathogens, apoptotic cell removal is generally considered as non-immunogenic and induces an anti-inflammatory response if the appropriate clearance mechanisms are present. In contrast, the uptake of necrotic cells is often associated with a pro-inflammatory response caused by the exposure of immunostimulatory molecules known as ‘danger’ signals (Gaipl et al., 2005; Krysko et al., 2006). Therefore, it is critical for the innate immune system to efficiently remove apoptotic and necrotic cells from the circulation and tissues to prevent the exposure of antigenic intracellular molecules as well as ‘danger’ signals that can promote the development of autoimmune diseases such as systemic lupus erythematosus (SLE) (Gaipl et al., 2005; Krysko et al., 2006).

Whilst many of the intracellular ‘eat-me’ signals of permeabilized/necrotic cells are not well characterized, a growing number of opsonins such as mannose-binding lectin (MBL), Ficolin-2, Ficolin-3, C-reactive protein (CRP), serum amyloid protein (SAP) and components of the classical complement pathway have been implicated in the clearance of permeabilized/necrotic cells (Gaipl et al., 2001; Bijl et al., 2003; Nauta et al., 2003; Ciurana et al., 2004; Hart et al., 2005; Honore et al., 2007; Jensen et al., 2007). In addition to these opsonins, histidine-rich glycoprotein (HRG), a member of the cystatin supergene-family, has been demonstrated recently to play an important role in the phagocytosis of late apoptotic (Gorgani et al., 2002) and necrotic cells (Jones et al., 2005b).
HRG is an abundant ~75 kDa multi-functional protein that is present in the plasma of many vertebrates. HRG has a multi-domain structure that allows the molecule to interact with multiple ligands including haem, Zn$^{2+}$, heparin, heparan sulfate (HS), plasminogen, fibrinogen, thrombospondin (TSP), IgG, C1q and Fcγ receptor (FcγR). The ability of HRG to interact with various ligands simultaneously has suggested that HRG can act as an adaptor molecule and regulate numerous biological processes such as immune complex/permeabilized cell/pathogen clearance, angiogenesis, cell adhesion, coagulation and fibrinolysis (see Section 1.1). In this chapter, the molecular components that are involved in HRG-mediated necrotic cell uptake have been characterized in detail, with HRG-mediated phagocytosis of necrotic cells resulting in the release of pro-inflammatory cytokines, such as TNF and IL-8, by monocytes.

3.3 Results

3.3.1 A specific subclass of human IgG co-purifies with plasma-derived HRG

Human HRG has been shown previously to bind strongly to human IgG (Gorgani et al., 1997; Gorgani et al., 1999c) and immune complexes (Gorgani et al., 1997; Gorgani et al., 1999b; Gorgani et al., 1999a). Therefore, it was important to validate the purity of plasma-derived HRG (HRG$^P$) prior to any experiments, especially those involving cells expressing FcγR. Initial experiments were performed using Western blotting and ELISA to determine the presence of IgG in HRG purified from human plasma. It was found that a relatively small amount of IgG was present in HRG$^P$, which could be removed from HRG$^P$ via Protein G column absorption to yield plasma-derived IgG Depleted HRG (HRG$^{PD}$) preparations (Fig. 3.1A and B). In order to further characterize the co-purified IgG isolated from HRG$^P$ (termed IgG$^{HRG}$), IgG$^{HRG}$ was eluted from the Protein G column and found to be essentially free of HRG (Fig. 3.1C). To further verify the purity of HRG$^{PD}$ and IgG$^{HRG}$, these preparations were examined by Coomassie Brilliant Blue protein staining following SDS-PAGE or by Western blotting of biotinylated samples of each preparation under denaturing and reducing conditions. No additional protein bands
Figure 3.1. IgG co-purifies with plasma-derived HRG. (A) Western blot analysis of the IgG content of human plasma-derived HRG (HRG\textsuperscript{p}) (3 μg) and plasma-derived IgG-Depleted HRG (HRG\textsuperscript{PID}) (3 μg), with a human IgG1κ myeloma (0.5 μg) being included as a positive control. (B) Analysis of the IgG content of HRG\textsuperscript{p} and HRG\textsuperscript{PID} preparations by ELISA using wells pre-coated with 1 μg/ml of each HRG preparation prior to blocking with 3% BSA. Error bars represent standard error of the mean (SEM) (n = 3). (C) Presence of HRG and IgG in preparations of HRG\textsuperscript{PID} (0.5 μg) and co-purified IgG isolated from HRG\textsuperscript{p} (IgG\textsuperscript{HRG}) (0.5 μg) as determined by Western blot analysis. (D) Further confirmation of the purity of HRG\textsuperscript{PID} (1 μg) and IgG\textsuperscript{HRG} (1 μg) by SDS-PAGE, in which protein bands were detected by 0.2% Coomassie Brilliant Blue stain (protein stain) or by Western blotting of biotinylated sample of each preparation (1.5 μg) using HRP-conjugated streptavidin. (E) Estimation of the content of IgG in a HRG\textsuperscript{p} preparation by densitometry analysis of Western blots, using pooled normal human IgG H-chain detection (upper panel) to construct a standard curve (lower panel).
were apparent in the HRG\textsuperscript{PID} and IgG\textsuperscript{HRG} preparations apart from the expected proteolytic fragments of HRG (see Chapter 4) and the H- and L-chains of human IgG, respectively (Fig. 3.1D). The amount of IgG present in HRG\textsuperscript{P} was estimated to be approximately 1-4% of HRG\textsuperscript{P} (w/w, 3 different preparations of HRG\textsuperscript{P} isolated from different plasma samples), based on densitometry analysis of Western blots for human IgG H-chain content (Fig. 3.1E).

Since HRG was previously reported to bind preferentially to human IgG myeloma proteins containing specific H- and L-chain subclasses (Gorgani \textit{et al.}, 1999c), the H- and L-chain composition of IgG\textsuperscript{HRG} was further investigated. Initially, the H-chain subclass composition of normal human IgG and IgG\textsuperscript{HRG} was compared using a human IgG subclass profile ELISA Kit. It was found that IgG\textsuperscript{HRG} preparations consisted predominantly of IgG2 (84.4%) and some IgG1 (12.7%), which is in striking contrast to the proportion of IgG subclasses present in pooled normal human IgG preparations in which IgG1 predominates (Fig. 3.2A). Furthermore, IgG\textsuperscript{HRG} preparations were found to contain a higher proportion of $\kappa$ L-chains to $\lambda$ L-chains than normal human IgG (Fig. 3.2B, C and D). Collectively, these results indicate that a specific subclass of IgG, namely IgG2$\kappa$, co-purifies with HRG\textsuperscript{P}, implying that the interaction between HRG and IgG may depend on the H- and L-chain composition of the IgG.

To further examine the interaction between HRG and the co-purified IgG, direct binding assays were performed by ELISA. Interestingly, HRG\textsuperscript{PID} preferentially bound to wells pre-coated with IgG\textsuperscript{HRG} than those pre-coated with IgG2$\kappa$ and IgG1$\kappa$ myeloma proteins (Fig. 3.3A). These results suggest that the interaction between HRG\textsuperscript{PID} and IgG\textsuperscript{HRG} may also depend on other factors such as the antigen binding properties or glycosylation state of IgG\textsuperscript{HRG}, rather than simply the H- and L-chain composition of IgG\textsuperscript{HRG}. Although HRG\textsuperscript{PID} could potentially bind IgG\textsuperscript{HRG} and the IgG2$\kappa$ myeloma via different mechanisms, basic pH and the presence of Zn$^{2+}$ or hemin, treatments that are known to modify the ligand binding capacity of HRG (Jones \textit{et al.}, 2005a), were equally effective at inhibiting the interaction between HRG\textsuperscript{PID} and IgG\textsuperscript{HRG} or the IgG2$\kappa$ myeloma (Fig. 3.3B, C and D).
Figure 3.2. IgG co-purified with HRG consists predominantly of IgG2 H-chains and κ L-chains. (A) Analysis of the proportion of different IgG subclasses present in IgG\textsubscript{HRG} and pooled normal human IgG preparations as determined by a human IgG subclass profile ELISA kit (Zymed\textsuperscript{®}). (B) Estimation of the amount of κ L-chain present in IgG\textsubscript{HRG} by densitometry analysis of Western blots, using detection of a human κ L-chain IgG4 myeloma (upper panel) to construct a standard curve (lower panel). (C) Estimation of the amount of λ L-chain present in IgG\textsubscript{HRG} by densitometry analysis of Western blots, using detection of a human λ L-chain IgG4 myeloma (upper panel) to construct a standard curve (lower panel). (D) Comparison of the relative amount of κ and λ L-chains present in pooled normal human IgG and IgG\textsubscript{HRG}.
Figure 3.3. HRG<sup>PID</sup> preferentially binds IgG<sup>HRG</sup>, an interaction that can be modulated by pH, Zn<sup>2+</sup> and hemin. (A) Analysis of the ability of HRG<sup>PID</sup> to bind different IgG preparations by ELISA using wells pre-coated with 2 µg/ml of IgG<sup>HRG</sup>, human IgG2κ or IgG1κ myeloma proteins. Error bars represent SEM (n = 3). Effect of (B) pH, (C) Zn<sup>2+</sup>, and (D) hemin on HRG<sup>PID</sup> binding to IgG<sup>HRG</sup> and a human IgG2κ myeloma was also examined by ELISA, with wells being pre-coated with 2 µg/ml of IgG<sup>HRG</sup> or a human IgG2κ myeloma and then analyzed for HRG<sup>PID</sup> (2 µg/ml) binding under the different conditions. Error bars represent SEM (n = 3).
3.3.2 HRG tethers the co-purified IgG to permeabilized/necrotic cells

Based on the above observation that a specific subclass of IgG predominately co-purifies with HRG\textsuperscript{p}, the importance of this co-purified IgG in previously reported functions of HRG\textsuperscript{p} was further investigated. HRG has been shown recently to bind strongly to permeabilized apoptotic (Gorgani \textit{et al.}, 2002) and necrotic cells (Jones \textit{et al.}, 2005b), and plays a vital role in dying/dead cell removal by phagocytes. To examine whether the presence of IgG in HRG\textsuperscript{p} preparations can influence the binding of HRG to viable and necrotic cells, Jurkat T cells were induced to be necrotic by exposure to hyperthermic condition (56°C) for 30 mins. The necrotic nature of the heat killed Jurkat T cells was validated by flow cytometry on the basis of cell size (forward scatter, FSC), cell granularity (side scatter, SSC), the ability to take up the DNA binding dye Hoechst 33258 and the exposure of phosphatidylserine (PS), detected via annexin V-PE staining. As expected, necrotic cells were more granular than viable cells (Fig. 3.4A) and >95% of the heat treated cells stained positive for Hoechst 33258 and annexin V-PE (Fig. 3.4B), which indicates that the cell membrane of the necrotic cells is permeabilized. In contrast, >92% of the viable cells stained negative for Hoechst 33258 and annexin V-PE (Fig. 3.4B), consistent with the cell membrane of the viable cells remaining intact. In agreement with previous studies, HRG in both the HRG\textsuperscript{p} and HRG\textsuperscript{PID} preparations (100 μg/ml) bound strongly and to a similar extent to necrotic cells, binding being 3-4 fold higher than to viable cells (Fig. 3.4C and D), suggesting that the presence or absence of the co-purified IgG had no major effect on HRG binding to either viable or necrotic cells. Furthermore, confocal laser scanning microscopy (CLSM) was used to visualize the binding of HRG\textsuperscript{PID} (100 μg/ml) to viable (Fig. 3.4E) and necrotic cells (Fig. 3.4F). Consistent with Jones \textit{et al.} (2005b), HRG\textsuperscript{PID} binding was localized to the cell surface of viable cells, possibly binding to cell surface HS, whereas necrotic cells exhibited an intracellular cytoplasmic binding pattern.

Strikingly, the co-purified IgG in the HRG\textsuperscript{p} preparation (100 μg/ml) was tethered specifically to necrotic but not to viable cells, with HRG\textsuperscript{PID} (100 μg/ml) being included
Figure 3.4. Analysis of the ability of HRG\textsuperscript{P} and HRG\textsuperscript{PID} to bind to viable and necrotic cells. Analysis of viable and necrotic (56°C for 30 mins) Jurkat T cells by flow cytometry on the basis of (A) forward scatter (FSC) and side scatter (SSC), and (B) Hoechst 33258 and annexin V-PE staining to determine viable, early apoptotic and permeabilized/necrotic cells. (C) Ability of HRG in HRG\textsuperscript{P} or HRG\textsuperscript{PID} preparations (100 μg/ml) to bind to either viable or necrotic cells as detected by flow cytometry using a mouse anti-human HRG mAb (HRG-4) and a PE-conjugated sheep F(ab')\textsubscript{2} anti-mouse Ig Ab. Representative flow cytometry histograms are shown, with filled histograms representing primary and secondary Ab only control, and open blue and red histograms representing HRG binding when HRG\textsuperscript{P} and HRG\textsuperscript{PID} preparations, respectively, were used. (D) Quantitative comparison of HRG binding to viable and necrotic cells, based on the data from (C), with data being expressed as fold binding above background mean fluorescence intensity (MFI). Error bars represent SEM (n = 3). Cellular localization of HRG\textsuperscript{PID} (100 μg/ml) bound to (E) viable or (F) necrotic cells as determined by confocal laser scanning microscopy (CLSM) using a mouse anti-human HRG mAb (HRG-4) and a FITC-conjugated sheep F(ab')\textsubscript{2} anti-mouse Ig Ab. ***, \( P < 0.001 \).
as a negative control and showing no binding of IgG to either viable or necrotic cells (Fig. 3.5A and B). Similar to HRG\textsuperscript{PID} (see Fig. 3.4F), the binding of both HRG and the co-purified IgG in the HRG\textsuperscript{P} preparation (100 μg/ml) were localized predominantly to the cytoplasm, and possibly even clustered on the surface, of necrotic cells (Fig. 3.5C and D). Based on these results, the ability of the co-purified IgG to bind to necrotic cells, either in the presence or absence of HRG, was investigated. IgG\textsuperscript{HRG} alone exhibited some binding to necrotic cell but this binding was markedly enhanced when a 1:1 molar ratio of HRG was present (Fig. 3.5E). However, the presence of a large molar excess of HRG\textsuperscript{PID} was able to significantly inhibit HRG-IgG complexes binding to necrotic cells, presumably by competing for HRG binding sites on necrotic cells (Fig. 3.5F). Collectively, these data suggest that HRG can mediate the binding of the co-purified IgG exclusively to necrotic but not to viable cells.

To examine whether the binding of HRG-IgG complexes to necrotic cells is limited only to heat induced necrotic cells, Jurkat T cells were permeabilized by three additional methods namely, (1) culturing the cells in serum free medium conditions for 48 hrs to induce late apoptotic and secondary necrotic cells, (2) exposure of the cells to mechanical damage via one freeze/thaw cycle, and (3) chemically permeabilizing the cells by exposure to the detergent Triton X-100 (0.05%). Due to the high level of background mouse anti-human HRG mAb (HRG-4) and secondary Ab binding to permeabilized (Hoechst 33258 positive) serum starved cells and freeze/thawed cells, it is unclear whether there are any substantial differences in HRG binding to permeabilized and non-permeabilized cells (Hoechst 33258 negative) generated using these two methods (Fig. 3.6A and B). Nevertheless, it is evident that HRG-IgG complexes in HRG\textsuperscript{P} (100 μg/ml) bound exclusively to permeabilized cells generated by all three methods (Fig. 3.6A, B and C), with the Triton X-100 permeabilized cells giving the clearest results. These data suggest that both apoptotic and heat induced cell death are not required to generate ligand(s) for HRG and HRG-IgG complex binding.
Figure 3.5. HRG mediates the binding of the co-purified IgG to necrotic cells. (A) Detection of IgG binding to viable and necrotic cells incubated with HRGP or HRGPD preparations (100 μg/ml) as determined by flow cytometry using a PE-conjugated goat F(ab')2 anti-human IgG γ-chain Ab. Representative flow cytometry histograms are shown, with filled histograms representing Ab only control and open blue and red histograms representing IgG binding when HRGP and HRGPD preparations, respectively, were used. (B) Quantitative comparison of IgG binding to viable and necrotic cells, based on the data from (A). Cellular localization of (C) HRG and (D) IgG bound to necrotic cells following incubation with HRGP (100 μg/ml). HRG and IgG binding to necrotic cells was determined by CLSM using a mouse anti-human HRG mAb (HRG-4) and a PE-conjugated sheep F(ab')2 anti-mouse Ig Ab or a PE-conjugated goat F(ab')2 anti-human IgG γ-chain Ab, respectively. (E) Quantitative comparison of IgGHRG (2 μg/ml) binding to necrotic cells either alone or in the presence of an approximately 1:1 molar ratio of HRG. (F) Effect of increasing concentrations of HRGPD (as molar ratio of HRGPD/IgGHRG) on the binding of IgGHRG to necrotic cells. IgG binding in (E) and (F) was determined by flow cytometry as described in (A). Data in (B), (E) and (F) are expressed as fold binding above background MFI, with error bars representing SEM (n = 3). *, P < 0.05. **, P < 0.01. ***, P < 0.001.
Figure 3.6. Comparison of the ability of HRG and HRG-IgG complexes to bind to non-permeabilized and permeabilized cells generated using different methods. Flow cytometry detection of HRG and HRG-IgG complexes binding to Hoechst 33258 negative (non-permeabilized) and positive (permeabilized) cells generated using three different methods. Jurkat T cells were (A) cultured in serum-free RPMI-1640 media for 48 hrs to induce late apoptotic and secondary necrotic cells, (B) exposed to one freeze/thaw cycle or (C) treated with 0.05% Triton X-100, and then incubated with 100 μg/ml of HRG. HRG and IgG binding was analyzed by flow cytometry as described in Figure 3.4 and 3.5, respectively. Representative flow cytometry histograms are shown, with filled histograms representing appropriate Ab only control (mouse anti-human HRG mAb, HRG-4, and PE-conjugated sheep F(ab')2 anti-mouse Ig Ab or PE-conjugated goat F(ab')2 anti-human IgG γ-chain Ab) and open black histograms representing HRG or IgG binding.
3.3.3 HRG and the co-purified IgG function co-operatively to facilitate phagocytosis of necrotic cells

Recently, HRG has been shown to act as an opsonin and specifically aid the phagocytosis of necrotic but not viable or early apoptotic cells (Jones et al., 2005b). Therefore, the ability of HRG to tether the co-purified IgG exclusively to necrotic cells (as shown above in Section 3.3.2) may potentially aid the uptake of permeabilized/necrotic cells by phagocytes. Comparable to previous studies, HRG\textsuperscript{P} (100 µg/ml) enhanced the removal of necrotic cells by THP-1 cells at levels 3-4 fold higher than the necrotic cells only control or when the phagocytic assay was performed in the presence of the negative control protein OVA (100 µg/ml) (Fig. 3.7A and B). The ability of HRG\textsuperscript{P} to enhance necrotic cell uptake was completely abolished by performing the phagocytic assay at 4°C or in the presence of the cytoskeleton disrupting agent cytochalasin-D (Cyto-D) at 37°C (Fig. 3.7B), suggesting that HRG\textsuperscript{P} is not simply enhancing the binding of necrotic cells to phagocytes. It is important to note that all phagocytic assays in this study were performed in the presence of 0.1% BSA rather than 10% heat inactivated fetal calf serum (HI-FCS) to allow direct examination of the ability of HRG\textsuperscript{P} to enhance necrotic cell removal and eliminate the involvement of a diverse array of serum proteins that may affect phagocytosis and subsequent phagocytosis-inhibition assays.

To investigate whether the presence of the co-purified IgG in HRG\textsuperscript{P} preparations can influence necrotic cell clearance, HRG\textsuperscript{PID} (100 µg/ml) and IgG\textsuperscript{HRG} (2 µg/ml) were either added independently or together to the phagocytic assay. Interestingly, HRG\textsuperscript{PID} alone only induced a small increase in phagocytosis (<1.5 fold) above the necrotic cells only control and IgG\textsuperscript{HRG} alone had no opsonic effects (Fig. 3.7C). In contrast, the presence of both HRG\textsuperscript{PID} and IgG\textsuperscript{HRG} markedly enhanced necrotic cell phagocytosis to levels ~2.5 fold higher than the necrotic cells only control (Fig. 3.7C). However, the reconstituted mixture of HRG\textsuperscript{PID} and IgG\textsuperscript{HRG} was not as efficient as unfractionated HRG\textsuperscript{P} (100 µg/ml) at enhancing necrotic cell phagocytosis (Fig. 3.7C), this difference possibly being due to the separation of HRG\textsuperscript{PID} and IgG\textsuperscript{HRG} affecting their functional...
Figure 3.7. HRG and the co-purified IgG function co-operatively to facilitate phagocytosis of necrotic cells. Phagocytic assays were performed using the monocytic cell line THP-1 and necrotic Jurkat T cells (56°C for 30 mins) labeled with PKH26 or CFSE, respectively. THP-1 cells were mixed with necrotic cells at a ratio of 1:10 and incubated at 37°C for 60 mins before analysis by flow cytometry. (A) Ability of HRGβ (100 μg/ml) to enhance the phagocytosis of necrotic cells by THP-1 cells. Representative flow cytometry plot are shown for phagocytic assays performed in the presence or absence of HRGβ. Values in each gated area represent percentage of cells in the assay. (B) Effect of temperature (4 or 37°C) and cytoskeleton disruption (Cyto-D, 25 μM) on the ability of HRGβ (100 μg/ml) to mediate the uptake of necrotic cells by THP-1 cells. Ovalbumin (OVA) (100 μg/ml) is included as a negative control protein. The level of phagocytosis was determined as the percentage of PKH26 positive THP-1 cells that had ingested CFSE positive necrotic cells, with data being expressed as fold difference in the level of phagocytosis relative to the necrotic cells only control. Error bars represent SEM (n = 3). (C) Effect of HRGβ or HRGβ (100 μg/ml), and IgGHRG (2 μg/ml), human IgG κ myeloma (2 μg/ml) or pooled normal human IgG (2 μg/ml) either alone or in the presence of HRGβ (100 μg/ml) on the phagocytosis of necrotic cells by THP-1 cells. Data are expressed as fold difference in the level of phagocytosis relative to the necrotic cells only control, with error bars representing SEM (n = 3). (D) Effect of 3 preparations (#A, #B and #C) of HRGβ or HRGβ (100 μg/ml) isolated from different plasma samples on the uptake of necrotic cells by THP-1 cells. Data are expressed as fold difference in the level of phagocytosis relative to the necrotic cells only control. NS, not significant. *, P < 0.05. **, P < 0.01. ***, P < 0.001.
activity. These data, nevertheless, suggest that HRG\textsuperscript{PID} and IgG\textsuperscript{HRG} can work co-operatively to aid the removal of necrotic cells. In contrast, the presence of either a human IgG2\kappa myeloma protein (2 \mu g/ml) or pooled normal human IgG (2 \mu g/ml) alone or together with the HRG\textsuperscript{PID} preparation (100 \mu g/ml) had no apparent enhancing effect on necrotic cell uptake (Fig. 3.7C), implying that a specific population of IgG molecules is needed to function co-operatively with HRG to aid the removal of necrotic cells. Furthermore, performing phagocytic assays using 3 different preparations (#A, #B and #C) of HRG\textsuperscript{P} or HRG\textsuperscript{PID} (100 \mu g/ml) isolated from different plasma samples (Fig. 3.7D), it was evident that the presence of the co-purified IgG in the HRG\textsuperscript{P} preparations is absolutely essential for enhanced necrotic cell clearance.

3.3.4 The role of Fc\gammaRI and Fc\gammaRIIA in HRG\textsuperscript{P}-mediated uptake of necrotic cells

It has been reported previously that HRG can facilitate the clearance of late apoptotic cells by human monocyte-derived macrophages (HMDM) via a Fc\gammaRI-dependent mechanism (Gorgani \textit{et al.}, 2002). Thus, the role of Fc\gammaR in HRG\textsuperscript{P}-induced phagocytosis of necrotic cells was investigated. Prior to performing phagocytosis-inhibition assays, the sub-optimal concentration of HRG\textsuperscript{P} required to enhance necrotic cell uptake was estimated to be between 50 and 100 \mu g/ml (Fig. 3.8A), with a concentration of 75 \mu g/ml of HRG\textsuperscript{P} being chosen for subsequent Fc\gammaRI and Fc\gammaRIIA blocking experiments. When a human IgG1\kappa myeloma was added to the phagocytic assay to saturate the IgG binding sites of Fc\gammaRI on THP-1 cells, a concentration dependent and, ultimately, complete inhibition of HRG\textsuperscript{P}-mediated necrotic cell uptake was observed, whereas the control protein OVA had no significant effects on phagocytosis (Fig. 3.8B). In order to confirm that Fc\gammaRI was involved in the enhanced clearance of necrotic cells by HRG\textsuperscript{P}, phagocytic assays were performed in the presence of saturating concentrations of a F(ab\textsuperscript{\prime})\textsubscript{2} anti-human Fc\gammaRI blocking mAb (10.1). This treatment resulted in complete inhibition of HRG\textsuperscript{P}-mediated uptake of necrotic cells (Fig. 3.8C). Collectively, these data suggest that Fc\gammaRI plays a key role in the HRG\textsuperscript{P}-mediated phagocytosis of necrotic cells by THP-1 cells.
Figure 3.8. HRG\textsuperscript{p} enhances phagocytosis of necrotic cells via a Fc\gammaRI-dependent mechanism. (A) Effect of increasing concentrations of HRG\textsuperscript{p} (6.25-200 \mu g/ml) on the phagocytosis of necrotic cells by THP-1 cells. (B) Effect of a human IgG1\kappa myeloma on HRG\textsuperscript{p} (75 \mu g/ml) -mediated uptake of necrotic cells, with OVA being included as a negative control. (C) Effect of a human Fc\gammaRI blocking mAb (mouse F(ab')\textsubscript{2} anti-human Fc\gammaRI mAb (10.1)) on the ability of HRG\textsuperscript{p} (75 \mu g/ml) to enhance the phagocytosis of necrotic cells. Data in each figure are expressed as fold difference in the level of phagocytosis relative to the necrotic cells only control. Error bars represent SEM (n = 3). NS, not significant. **, P < 0.01. ***, P < 0.001.
In addition to the high affinity IgG receptor, FcγRI, the monocyte-like cell line THP-1 also expresses the low affinity IgG receptor, FcγRIIA (Fleit and Kobasiuk, 1991). Although the above results suggest that FcγRI is the major FcγR on THP-1 cells involved in HRG^p-mediated necrotic cell uptake, FcγRIIA may also play a secondary role in this process. The ability of the co-purified IgG in HRG^p to bind to FcγRIIA was initially investigated using a glycosaminoglycan(s) (GAG) deficient CHO cell line (pgsA-745) stably transfected with the two different allelic forms of human FcγRIIA. These two FcγRIIA contain either a histidine residue or an arginine residue at amino acid position 131 (termed FcγRIIA-his131 or FcγRIIA-arg131, respectively), with the polymorphism altering the binding affinity of FcγRIIA for human IgG2 (Warmerdam et al., 1991). As shown in Figure 3.9A, pgsA-745 cells stably transfected with either FcγRIIA-his131 or FcγRIIA-arg131 expressed similar cell surface levels of FcγRIIA, as detected by a F(ab')2 anti-human FcγRIIA mAb (8.26), whereas untransfected pgsA-745 cells showed no expression of FcγRIIA. Consistent with previous studies which showed that human IgG2 preferentially binds to FcγRIIA-his131 but not to FcγRIIA-arg131 (Warmerdam et al., 1991), the co-purified IgG from HRG^p preparations (consist of predominately IgG2) bound significantly stronger to pgsA-745 cells expressing FcγRIIA-his131 than to cells expressing FcγRIIA-arg131 (Fig. 3.9B). Although THP-1 cells are known to be homozygous for the FcγRIIA-his131 allotype (Tebo et al., 2002), a F(ab') anti-human FcγRIIA blocking mAb (IV.3) at saturating concentrations only had a small, but statistically significant, inhibitory effect on HRG^p-aided uptake of necrotic cells (Fig. 3.9C), suggesting that FcγRIIA may play a minor role in this process. Nevertheless, polymorphisms in FcγRIIA may influence HRG^p-mediated necrotic cell clearance by other phagocytic cells such as unstimulated neutrophils, which express mainly the low affinity IgG receptors, FcγRIIA and FcγRIII (Repp et al., 1991).

Furthermore, it is important to note that FcγRI and FcγRIIA blocking mAbs have been shown previously to be ineffective in reducing necrotic cell uptake enhanced by HRG^p (Jones et al., 2005b). The conflicting data observed between the current study and the
Figure 3.9. FcyRIIA plays a minor role in HRG\textsuperscript{P}-mediated phagocytosis of necrotic cells. (A) Cell surface expression of human FcyRIIA by the glycosaminoglycan(s) (GAG) deficient CHO cell line (pgsA-745) stably transfected with human FcyRIIA containing either an arginine residue or a histidine residue at amino acid position 131 (FcyRIIA-arg131 and FcyRIIA-his131, respectively), with untransfected pgsA-745 cells being included as a negative control. Human FcyRIIA expression was detected by flow cytometry using a mouse F(ab')\textsuperscript{2} anti-human FcyRIIA mAb (8.26) and a PE-conjugated sheep F(ab')\textsuperscript{2} anti-mouse Ig Ab. Representative flow cytometry histograms are shown, with filled histograms representing secondary Ab only control, and open black histograms representing cell surface expression of human FcyRIIA. (B) Ability of HRG-IgG complexes in a HRG\textsuperscript{P} preparation (100 µg/ml) to binding to pgsA-745 cells stably transfected with either human FcyRIIA-arg131 or FcyRIIA-his131, with untransfected pgsA-745 cells being included as a negative control. IgG binding was determined by flow cytometry as described in Figure 3.5, with data being expressed as fold binding above background MFI. Error bars represent SEM (n = 3). (C) Effect of a human FcyRIIA blocking mAb (mouse F(ab') anti-human FcyRIIA mAb (IV.3)) on HRG\textsuperscript{P} (75 µg/ml) -mediated phagocytosis of necrotic cells, with data being expressed as fold difference in the level of phagocytosis relative to the necrotic cells only control. Error bars represent SEM (n = 3). NS, not significant. *, P < 0.05. ***, P < 0.001.
previous studies by Jones et al. (2005b) is likely to be a result of different concentrations of blocking mAbs being used in the phagocytosis-inhibition assays, with saturating concentrations of FcγRI and FcγRIIA blocking mAbs being used in the current study.

### 3.3.5 Ability of HRG and HRG-IgG complexes to bind to THP-1 cells

Previously, studies have suggested that HRG can interact directly with FcγRI on both THP-1 cells (Gorgani et al., 1999b) and HMDM (Gorgani et al., 2002), and modulate the binding of monomeric IgG and immune complexes to THP-1 cells (Gorgani et al., 1999b). However, it should be noted that the purification method used in all these studies to isolate HRG from human plasma did not eliminate the presence of IgG in the HRG<sup>P</sup> preparations and casts doubts on the conclusions drawn. Thus, it was important to determine whether the reported ability of HRG to interact with FcγR on THP-1 cells is actually mediated by the co-purified IgG present in the HRG<sup>P</sup> preparations, rather than directly via HRG. As shown in Figure 3.10A, THP-1 cells expressed both FcγRI and FcγRIIA and bound a human IgG1κ myeloma efficiently. Like most cell types, THP-1 cells also expressed cell surface HS (Fig. 3.10A), which is an important cell surface ligand of HRG (Jones et al., 2004b). In order to examine the direct interaction of HRG with THP-1 cells, biotinylated HRG<sup>PID</sup> (100 μg/ml) and PE-conjugated streptavidin were used in cell surface binding assays, with streptavidin being used to avoid any complications arising from the possible binding of HRG-specific detection antibodies to FcγR on THP-1 cells. The biotinylated HRG<sup>PID</sup> bound strongly to THP-1 cells and the binding was completely inhibited by the presence of heparin (12.5 kDa, 50 μg/ml) (Fig. 3.10B). However, pre-coating THP-1 cells with either a human IgG1κ myeloma, a human FcγRI blocking mAb (10.1) or a human FcγRIIA blocking mAb (8.26) at saturating concentrations had no apparent effect on the binding of biotinylated HRG<sup>PID</sup> (Fig. 3.10B). These data suggest that the interaction between HRG and THP-1 cells is likely to be mediated via cell surface HS and not via FcγR (see also Section 3.3.9 below).
In order to investigate the interaction between HRG-IgG complexes and THP-1 cells, THP-1 cells were incubated with HRG\(^\text{P}\) (100 µg/ml) and then analyzed for human IgG binding. HRG-IgG complexes bound strongly to THP-1 cells and this interaction was significantly reduced by pre-coating THP-1 cells with saturating concentrations of a human Fc\(\gamma\)RI blocking mAb (10.1) but not by a human Fc\(\gamma\)RIIA blocking mAb (8.26) prior to HRG\(^\text{P}\) exposure (Fig. 3.10C). Thus, the ability of HRG to interact with Fc\(\gamma\)R on THP-1 cells, in particular Fc\(\gamma\)RI, is likely to be mediated via the co-purified IgG as an intermediate adaptor molecule. However, since the co-purified IgG contains mainly IgG2, the weakest Fc\(\gamma\)RI binding IgG subclass (Canfield and Morrison, 1991), it is surprising that Fc\(\gamma\)RI was the key Fc\(\gamma\)R in mediating the co-purified IgG binding to THP-1 cells (Fig. 3.10C). Nevertheless, these results are consistent with the data presented earlier (see Section 3.3.2, 3.3.3 and 3.3.4) indicating that HRG-IgG complexes are able to bridge THP-1 cells to necrotic cells, with the HRG component tethering the co-purified IgG to necrotic cells and the co-purified IgG binding to Fc\(\gamma\)RI on THP-1 cells and triggering phagocytosis of the necrotic cells.

Previous studies by Gorgani et al. (1999b) also demonstrated that pre-coating THP-1 cells with HRG was able to block monomeric IgG binding and these data were interpreted as being due to the direct interaction of HRG with Fc\(\gamma\)RI on the THP-1 cells. In an attempt to further clarify this issue, THP-1 cells were pre-treated with either HRG\(^\text{P}\) or HRG\(^{\text{PID}}\) preparations (100 µg/ml) and then analyzed for biotinylated human IgG1\(\kappa\) myeloma binding. Interestingly, only cells pre-coated with HRG\(^\text{P}\), but not with HRG\(^{\text{PID}}\), showed a reduction in biotinylated human IgG1\(\kappa\) myeloma binding (Fig. 3.10D and E), suggesting that the previously reported observations by Gorgani et al. (1999b) are likely to be a result of the presence of the co-purified IgG in HRG preparations purified from human plasma.
Figure 3.10. Analysis of the interactions of HRG and HRG-IgG complexes with THP-1 cells. (A) Cell surface expression of human FcyRI, FcyRIIA and HS by THP-1 cells or human IgG1κ binding by THP-1 cells was examined using a mouse F(ab')2 anti-human FcyRI mAb (10.1), a mouse F(ab')2 anti-human FcyRIIA mAb (8.26), a mouse anti-HS mAb (F58-10E4) or a human IgG1κ myeloma. Mouse mAb binding and human IgG1κ myeloma binding was detected by flow cytometry using a PE-conjugated sheep F(ab')2 anti-mouse Ig Ab and a PE-conjugated goat F(ab')2 anti-human IgG γ-chain Ab, respectively. Representative flow cytometry histograms are shown, with filled histograms representing the appropriate PE-conjugated Ab only control, and open black histograms representing human FcyRI, FcyRIIA and HS expression or human IgG1κ myeloma binding. (B) Effect of pre-coating THP-1 cells with a human FcyRI-specific blocking mAb (10.1, 40 μg/ml), a human FcyRIIA-specific blocking mAb (8.26, 40 μg/ml) or a human IgG1κ myeloma (200 μg/ml) on biotinylated HRG<sup>PID</sup> (100 μg/ml) binding to THP-1 cells. The effect of heparin (12.5 kDa, 50 μg/ml) on biotinylated HRG<sup>PID</sup> binding to THP-1 cells was also examined. Biotinylated HRG<sup>PID</sup> binding was detected by flow cytometry using PE-conjugated streptavidin. Representative flow cytometry histograms are shown, with filled histograms representing the PE-conjugated streptavidin only control and open blue and red histograms representing, respectively, biotinylated HRG<sup>PID</sup> binding in the absence or presence of the indicated treatments. (C) Effect of pre-coating THP-1 cells with a human FcyRI-specific mAb (10.1, 40 μg/ml) or a human FcyRIIA-specific mAb (8.26, 40 μg/ml) on the binding of HRG-IgG complexes in HRG<sup>P</sup> (100 μg/ml) to THP-1 cells. IgG binding was determined by flow cytometry as described in Figure 3.5, with data being expressed as fold binding above background MFI. Error bars represent SEM (n = 3). Effect of pre-coating THP-1 cells with (D) HRG<sup>P</sup> or (E) HRG<sup>PID</sup> on the binding of biotinylated human IgG1κ myeloma to THP-1 cells. Biotinylated IgG binding was determined by flow cytometry using PE-conjugated streptavidin, with data being expressed as fold binding above background MFI. NS, not significant. ***, P < 0.001.
A. THP-1 cells

B. ± F(ab')2 anti-FcyRI mAb (10.1) ± F(ab')2 anti-FcyRIIA mAb (8.26)

C. IgG binding (Fold above background MFI)

D. IgG binding (Fold above background MFI) vs Biotin-IgG1K (ng/ml)

E. IgG binding (Fold above background MFI) vs Biotin-IgG1K (ng/ml)

Chapter 3
3.3.6 HRGP has little effect on C1q binding to necrotic cells

Complement component C1q, the initiator of the classical complement pathway, has been shown in a number of studies to play a vital role in apoptotic and necrotic cell clearance (Mevorach et al., 1998; Taylor et al., 2000; Ogden et al., 2001; Ciurana et al., 2004). C1q can bind to dying/dead cells and activate the classical complement pathway either via direct interaction with a yet to be identified ligand(s) exposed on dying/dead cells (Ogden et al., 2001) or through 'natural' IgM antibodies that bind specifically to dying/dead cells (Kim et al., 2002; Ogden et al., 2005; Quartier et al., 2005). Previously, studies have reported that C1q interacts directly with HRG (Gorgani et al., 1997) as well as IgG (Duncan and Winter, 1988). Thus, HRG and HRG-IgG complexes could potentially enhance C1q binding to necrotic cells. Initially, to examine whether C1q co-purifies with HRGP, C1q Western blotting analysis was performed and showed that no C1q could be detected in either the HRGP or IgGHRG preparations (Fig. 3.11A), suggesting that it is unlikely that C1q co-purifies with HRGP. Furthermore, low level binding of C1q to necrotic cells (~3 fold above background staining) was detected and the presence of HRGP but not HRGPID (100 μg/ml) was able to significantly increase the binding of C1q (Fig. 3.11B), indicating that HRG-IgG complexes in HRGP can aid C1q binding to necrotic cells. However, it is important to note that the enhancing effect of HRGP was very small, probably due to the inability of IgG2 (i.e. the main IgG subclass present in the co-purified IgG) to bind C1q efficiently (Bindon et al., 1988). Thus, HRG-IgG complexes are unlikely to play an important role in facilitating C1q binding to necrotic cells under normal physiological conditions. Nevertheless, complement components and HRG may work in concert in vivo to aid necrotic cell clearance by phagocytes.

3.3.7 Ability of HRGP to enhance necrotic cell phagocytosis is modulated by Zn2+, hemin and heparin

As described in Section 1.1, HRG is a multi-domain protein that can bind to a wide variety of ligands via different mechanisms (see Section 1.1 and Fig. 1.2). Therefore, the
Figure 3.11. HRG<sup>P</sup> has little effect on C1q binding to necrotic cells. (A) Western blot analysis of the human C1q content of HRG<sup>PID</sup> or IgG<sup>HRG</sup> (300 ng), with human C1q (300 ng) being included as a positive control. (B) Effect of HRG<sup>P</sup> or HRG<sup>PID</sup> (100 μg/ml) on human C1q (100 μg/ml) binding to necrotic Jurkat T cells. C1q binding was determined by flow cytometry using a mouse anti-human C1q mAb (MHC5B9) and a PE-conjugated sheep F(ab')<sub>2</sub> anti-mouse Ig Ab. Data are expressed as fold binding above background MFI, with error bars representing SEM (n = 3). NS, not significant. *, P < 0.05.
effect of a range of HRG ligands (e.g. Zn$^{2+}$, haem and heparin) on the ability of HRG$^P$ (100 μg/ml) to enhance necrotic cell clearance was investigated. Firstly, the presence of physiological concentrations of Zn$^{2+}$ (~20 μM) significantly potentiated necrotic cell uptake mediated by HRG$^P$, whereas control phagocytic assays performed in the presence of 20 μM Zn$^{2+}$ showed a small but statistically significant reduction in phagocytosis (Fig. 3.12A). In contrast, the presence of hemin (the Fe$^{3+}$ oxidation product of haem) significantly reduced necrotic cell removal mediated by HRG$^P$, but had no effect on control necrotic cell uptake (Fig. 3.12B). Since both Zn$^{2+}$ and hemin are thought to bind to the HRR of HRG (Morgan, 1981; Katagiri et al., 1987) and, based on their small size, they are unlikely to have any significant steric effects on the ligand binding properties of HRG, these results suggest that the HRR itself or a ligand-induced conformational change in the HRG molecule following Zn$^{2+}$ or hemin binding is important in HRG$^P$-mediated phagocytosis of necrotic cells. Although the molecular mechanisms underpinning the effect of Zn$^{2+}$ are unclear, enhanced Zn$^{2+}$ levels at sites of tissue injury due to degranulating platelets (Aktulga, 1974; Gorodetsky et al., 1993) may aid the removal of dying/dead cells via HRG.

In contrast to the previous studies by Jones et al. (2005b), the presence of heparin (12.5 kDa) at the highest concentration of 20 μg/ml markedly inhibited HRG$^P$-mediated phagocytosis of necrotic cells below control levels (Fig. 3.12C). The conflicting data observed between the current study and the previous studies by Jones et al. (2005b), in which heparin (12.5 kDa) was ineffective in blocking HRG$^P$-mediated necrotic cell uptake, is likely to be a result of a discrepancy between the phagocytic assays used by the two studies. The presence of other serum proteins, in particular heparin-binding proteins, in the phagocytic assays used by Jones et al. (2005b) could have potentially masked the inhibitory effects of heparin. In addition, the inhibitory effect of heparin further supports the involvement of the HRR of HRG in necrotic cell clearance, since the HRR, in additional to the N1N2 domain of HRG (Jones et al., 2004b), represents a major heparin/HS binding site on HRG (Vanwildemeersch et al., 2006). Surprisingly, heparin (12.5 kDa) also significantly reduced necrotic cell uptake in the absence of
Figure 3.12. Effect of Zn$^{2+}$, hemin and heparin on the enhanced phagocytosis of necrotic cells induced by HRGP. Effect of (A) Zn$^{2+}$ (20 μM), (B) hemin or (C) heparin (12.5 kDa) on HRGP (100 μg/ml)-mediated phagocytosis of necrotic cells. Data are expressed as fold difference in the level of phagocytosis relative to the necrotic cells only control, with error bars representing SEM (n = 3).
HRG\(^P\) (Fig. 3.12C), suggesting that heparin can potentially disrupt the interaction between THP-1 cells and necrotic cells and/or directly modulate the phagocytic activity of the THP-1 cells. Nevertheless, since HRG is a strong heparin/HS binding protein (Heimburger \textit{et al.}, 1972; Lijnen \textit{et al.}, 1983; Borza and Morgan, 1998; Jones \textit{et al.}, 2004a), these results suggest that the presence of heparin during anticoagulation treatment, or following mast cell degranulation (Lindstedt and Kovanen, 2006), as well as the release of HS fragments from the extracellular matrix (ECM) at sites of tissue remodelling (Elkin \textit{et al.}, 2001; Li \textit{et al.}, 2005), may interfere with the ability of HRG to enhance necrotic cell removal.

### 3.3.8 Heparin and HS inhibit various interactions that are critical for HRG\(^P\)-mediated necrotic cell removal

The ability of heparin to inhibit HRG\(^P\)-mediated necrotic cell clearance by phagocytes (see above) has important implications, with heparin being routinely administrated clinically as an anticoagulant agent or released by activated mast cells to regulate various biological processes (Page, 1997). Since necrotic cell uptake enhanced by HRG\(^P\) involves complex interactions between the necrotic cell, HRG, the co-purified IgG and the phagocyte, the effect of heparin on these interactions was further examined. The presence of heparin (12.5 kDa) or HS markedly reduced HRG\(^P\) binding to both immobilized IgG\(^{HRG}\) and a human IgG\(_2\kappa\) myeloma as measured by ELISA (Fig. 3.13A and B). These data are consistent with earlier results, in which ligand binding can affect the interaction of HRG with IgG (see Section 3.3.1) and modulate the ability of HRG\(^P\) to enhance necrotic cell uptake (see Section 3.3.7). Importantly, these data also suggest that the binding of HRG to cell surface HS on viable cells may inhibit the ability of HRG to tether IgG to viable cells.

The effect of heparin on the binding of HRG and HRG-IgG complexes to necrotic Jurkat T cells and viable THP-1 cells was also investigated. The presence of heparin (12.5 kDa)
Figure 3.13. Heparin and HS inhibit the binding of HRG\(_{PID}^{HRG}\) to IgG\(_{HRG}^{HRG}\) and a human IgG2κ myeloma. Effect of (A) heparin (12.5 kDa) and (B) HS on the binding of HRG\(_{PID}^{HRG}\) (2 μg/ml) to IgG\(_{HRG}^{HRG}\) and a human IgG2κ myeloma was quantified by ELISA using wells pre-coated with 2 μg/ml of IgG\(_{HRG}^{HRG}\) or the human IgG2κ myeloma. Error bars represent SEM (n = 3).
at a concentration of 50 µg/ml significantly reduced the binding of both HRG and HRG-IgG complexes in HRG preparations (100 µg/ml) to necrotic cells by ~55% and ~70%, respectively (Fig. 3.14A, B and C). Consistent with previous studies by Jones et al. (2005b), these data suggest that the binding of heparin to HRG can partially reduce the ability of HRG to interact with its necrotic cell ligand(s). The reduction in HRG-IgG complexes binding to necrotic cells could also be the result of heparin interfering with the interaction between HRG and the co-purified IgG as described above. Alternatively, the HRG binding site on necrotic cells may be being blocked by heparin. To examine the latter, necrotic cells were pre-coated with heparin (12.5 kDa) and this treatment was found to have no significant effect on HRG binding (Fig. 3.14D), indicating that the inhibitory effect of heparin on the binding of HRG and HRG-IgG complexes to necrotic cells is likely to be mediated via the direct interaction of heparin with HRG.

In additional experiments, the ability of HRG to bind directly to viable THP-1 cells was investigated using a HRG preparation (100 µg/ml) and a F(ab')2 anti-human HRG mAb (HRG-4) to avoid the possible binding of HRG-IgG complexes and the HRG-specific detection antibodies to FcγR on the THP-1 cells. Consistent with earlier results using biotinylated HRG (see Section 3.3.5), the presence of heparin (12.5 kDa, 50 µg/ml) reduced the binding of HRG to viable THP-1 cells by >95% (Fig. 3.15A and B), suggesting that the binding of HRG to viable THP-1 cells is likely to be mediated via cell surface HS. Unexpectedly, the presence of heparin (12.5 kDa, 50 µg/ml) also had a small inhibitory effect on the binding of both HRG-IgG complexes in HRG preparations (100 µg/ml) and IgG (2 µg/ml) to viable THP-1 cells (Fig 3.15A, C and D), possibly due to steric inhibition of Fc-FcγR interactions by heparin-binding proteins on THP-1 cells. Collectively, these data show that the ability of heparin to inhibit necrotic cell uptake aided by HRG preparations may involve a number of different molecular mechanisms.
Figure 3.14. Effect of heparin on the binding of HRG and HRG-IgG complexes to necrotic Jurkat T cells. (A) Effect of heparin (12.5 kDa, 50 µg/ml) on the ability of HRG and HRG-IgG complexes in a HRG \( \text{P} \) preparation (100 µg/ml) to bind to necrotic cells. Representative flow cytometry histograms are shown, with filled histograms representing the appropriate Ab only control and open blue and red histograms representing HRG and IgG binding in the absence or presence of heparin (12.5 kDa), respectively. Quantitative comparison of (B) HRG and (C) IgG binding to necrotic cells based on the data from (A). (D) Quantitative comparison on HRG in a HRG \( \text{P} \) preparation (100 µg/ml) to bind to necrotic cells pre-coated with 20 or 50 µg/ml of heparin (12.5 kDa). HRG and IgG binding was detected by flow cytometry as described, respectively, in Figure 3.4 and 3.5. Data in (B), (C) and (D) are expressed as fold binding above background MFI. Error bars represent SEM (n = 3). NS, not significant. *, \( P < 0.05 \). **, \( P < 0.01 \). ***, \( P < 0.001 \).
Figure 3.15. Effect of heparin on the binding of HRG and HRG-IgG complexes to viable THP-1 cells. (A) Effect of heparin (12.5 kDa, 50 μg/ml) on the ability of HRG^{PD} (100 μg/ml), HRG-IgG complexes in a HRG^{P} preparation (100 μg/ml) and IgG^{HRG} (2 μg/ml) to bind to viable THP-1 cells. HRG binding was detected using a mouse F(ab') 2 anti-human HRG mAb (HRG-4) and a PE-conjugated sheep F(ab') 2 anti-mouse Ig Ab. IgG binding was detected by flow cytometry as described in Figure 3.5. Representative flow cytometry histograms are shown, with filled histograms representing the appropriate Ab only control and open blue and red histograms representing HRG and IgG binding in the absence or presence of heparin (12.5 kDa), respectively. Quantitative comparison of (B) HRG and (C, D) IgG binding to THP-1 cells based on the data from (A). Data in (B), (C), and (D) are expressed as fold binding above background MFI. Error bars represent SEM (n = 3). *, P < 0.05. **, P < 0.01. ***, P < 0.001.
3.3.9 The role of cell surface HS on phagocytes in HRG\(^p\)-mediated necrotic cell clearance

Heparin, apart from being a well characterized ligand of HRG (Jones et al., 2005a), can itself bind strongly to permeabilized cells and the HS component on the surface of macrophages has been proposed to facilitate the phagocytosis of dying cells (Gebska et al., 2002). Therefore, to elucidate whether cell surface HS on phagocytes is involved in necrotic cell clearance mediated via HRG\(^p\), viable THP-1 cells were treated with human platelet-derived heparanase to remove cell surface HS and the effect of this treatment on HRG binding and necrotic cell uptake was examined. Initially, the ability of heparanase to cleave cell surface HS from viable THP-1 cells and the re-expression of cell surface HS on these cells at 37\(^\circ\)C following heparanase treatment was investigated. As indicated in Figure 3.16A, cell surface HS on THP-1 cells was substantially removed by heparanase exposure and cell surface HS re-expression did not occur after incubation for 60 mins at 37\(^\circ\)C following heparanase treatment. As expected, HRG\(^{PD}\) (100 \(\mu\)g/ml) binding to THP-1 cells was markedly reduced by pre-treating THP-1 cells with human heparanase, the reduction in binding being comparable to that achieved by heparin inhibition (Fig. 3.16B), suggesting that HS is the key cell surface ligand for HRG on viable THP-1 cells. Interestingly, heparanase-treated THP-1 cells showed a significant reduction in necrotic cell uptake aided by HRG\(^p\) (100 \(\mu\)g/ml), whereas both untreated and heparanase-treated THP-1 cells exhibited a similar level of necrotic cell uptake in the absence of HRG\(^p\) (Fig. 3.16C and D). These data suggest that cell surface HS on THP-1 cells and HRG\(^p\) may act in concert to aid the disposal of necrotic cells, and the inhibitory effect of heparanase or heparin on necrotic cell uptake (as shown in Section 3.3.7) can potentially interfere with the ability of cell surface HS on THP-1 cells to facilitate phagocytosis. Thus, although cell surface HS on phagocytes alone may not be sufficient to trigger phagocytosis of necrotic cells, it is worth noting that these results do not eliminate the possibility that a residual amount of HS is present on THP-1 cells following heparanase treatment and is capable of aiding necrotic cell uptake via either HRG\(^p\)-dependent or -independent mechanisms.
Figure 3.16. Cell surface HS on phagocytic cells plays an important role in HRG<sup>P</sup>-mediated phagocytosis of necrotic cells. (A) Analysis of cell surface expression of HS on untreated THP-1 cells or THP-1 cells incubated at 37°C for 0, 30 or 60 mins following human platelet-derived heparanase treatment (4 μg/ml, 37°C, 60 mins), with HS being detected by flow cytometry as described in Figure 3.10. Data are expressed as fold binding above background MFI. (B) Effect of heparanase pre-treatment (4 μg/ml) at 37°C for 60 mins and heparin (12.5 kDa, 50 μg/ml) on the ability of HRG<sup>P</sup> (100 μg/ml) to bind to viable THP-1 cells. HRG binding was detected by flow cytometry as described in Figure 3.15. Representative flow cytometry histograms are shown, with filled histograms representing the appropriate Ab only control and open black histograms representing HRG binding. (C) Ability of heparanase-treated THP-1 cells to phagocytosis necrotic cells in the presence or absence of HRG<sup>P</sup> (100 μg/ml). Representative flow cytometry plot are shown for the different phagocytic assays. Values in each gated area represent percentage of cells in the assay. (D) Quantitative comparison of the phagocytosis of necrotic cells, based on the data from (C), with data being expressed as fold difference in the level of phagocytosis relative to the untreated THP-1 cells control. Error bars represent SEM (n = 3). NS, not significant. **, P < 0.01.
3.3.10 The role of necrotic cell HS in HRG<sup>P</sup>-mediated necrotic cell clearance

In addition to binding to HS on phagocytic cells, HRG can also bind to cell surface HS on necrotic cells and this interaction may play an important role in the ability of HRG<sup>P</sup> to enhance the disposal of necrotic cells. Thus, in order to examine the role of cell surface HS on necrotic cells in this process, necrotic Jurkat T cells were treated with human platelet-derived heparanase to remove exposed HS. HRG in HRG<sup>P</sup> preparations (100 µg/ml) bound to both untreated and heparanase-treated necrotic Jurkat T cells to a similar extent (Fig. 3.17A), suggesting that unlike viable cells (see Fig. 3.16), HS is not the key HRG ligand present on necrotic cells. Surprisingly, heparanase treatment actually enhanced the binding of HRG-IgG complexes in HRG<sup>P</sup> preparations (100 µg/ml) to necrotic Jurkat T cells (Fig. 3.17B). These data suggest that cell surface HS may actively interfere with the ability of HRG-IgG complexes to bind to the exposed cytoplasmic ligand(s) of necrotic cells. In additional experiments, heparin (12.5 kDa, 50 µg/ml) was equally effective in reducing the binding of HRG and HRG-IgG complexes in HRG<sup>P</sup> preparations (100 µg/ml) to both untreated and heparanase-treated necrotic Jurkat T cells (Fig. 3.17A and B), suggesting that heparin can block the HRG binding site for necrotic cells, even though the necrotic cell ligand is not HS. In phagocytic assays, heparanase treatment of necrotic Jurkat T cells had no apparent effect on either control or HRG<sup>P</sup>-enhanced uptake of necrotic cells (Fig 3.17C), indicating that necrotic cell clearance under control conditions or via HRG<sup>P</sup> is independent of HS expressed on the necrotic cells.

To further verify that cell surface HS on necrotic cells plays little or no role in necrotic cell uptake, the GAG deficient CHO cell line (pgsA-745), which expresses no detectable cell surface HS compared to the wild type HS-bearing CHO-K1 cells (Fig. 3.17D), was used as the necrotic target cells. HRG and HRG-IgG complexes in HRG<sup>P</sup> preparations (100 µg/ml) bound strongly to necrotic pgsA-745 cells and this binding was significantly
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Figure 3.17. Necrotic cell HS is not involved in the enhanced phagocytosis of necrotic cells induced by HRG\(\text{P}\). Effect of heparanase pre-treatment (4 \(\mu\)g/ml, 37°C, 60 mins) and heparin (12.5 kDa, 50 \(\mu\)g/ml) on the ability of (A) HRG and (B) HRG-IgG complexes in a HRG\(\text{P}\) preparation (100 \(\mu\)g/ml) to bind to necrotic Jurkat T cells. (C) Phagocytosis of untreated or heparanase-treated necrotic Jurkat T cells by THP-1 cells in the presence or absence of HRG\(\text{P}\) (100 \(\mu\)g/ml). (D) Cell surface expression of HS by viable CHO-K1 cells and a GAG deficient CHO cell line (pgsA-745), as detected by flow cytometry as described in Figure 3.10. Representative flow cytometry histograms are shown, with filled histograms representing secondary Ab only control and open black histograms representing HS expression. Effect of heparin (12.5 kDa, 50 \(\mu\)g/ml) on the ability of (E) HRG and (F) HRG-IgG complexes in a HRG\(\text{P}\) preparation (100 \(\mu\)g/ml) to bind to necrotic GAG deficient pgsA-745 cells. (G) Effect of heparin (12.5 kDa, 50 \(\mu\)g/ml) on HRG\(\text{P}\) (100 \(\mu\)g/ml) -mediated phagocytosis of necrotic pgsA-745 cells. For flow cytometry-based cell binding assays, HRG and IgG binding was detected as described in Figure 3.4 and 3.5, respectively, with data being expressed as fold binding above background MFI. Error bars represent SEM (n = 3). For phagocytic assays, data are expressed as fold difference in the level of phagocytosis relative to the untreated necrotic cells only control, with error bars representing SEM (n = 3). NS, not significant. **, \(P < 0.01\). ***, \(P < 0.001\).
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**A**

HRG binding (Fold above background MFI)

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IgG binding (Fold above background MFI)

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Fold differences in phagocytosis

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**D**

Viable CHO-K1 cells

Viable pgsA-745 cells

**E**

HRG binding (Fold above background MFI)

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IgG binding (Fold above background MFI)

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**G**

Fold differences in phagocytosis

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inhibited by heparin (12.5 kDa, 50 μg/ml) (Fig. 3.17E and F). Similar to necrotic Jurkat T cells, HRG$^P$ (100 μg/ml) efficiently enhanced the phagocytosis of necrotic HS-deficient pgsA-745 cells and the enhanced uptake mediated by HRG$^P$ was completely abolished by heparin (12.5 kDa, 20 μg/ml) (Fig. 3.17G). Collectively, these data support the view that cell surface HS on necrotic cells plays little or no role in HRG binding to necrotic cells or necrotic cell clearance via HRG$^P$. Furthermore, the major inhibitory effect of heparin is unlikely to be mediated through the blocking of HRG binding to cell surface HS on necrotic cells.

### 3.3.11 Hemin inhibits HRG binding to necrotic cells and heparin

Similar to the effect of heparin as described above (see Section 3.3.8 and 3.3.10), hemin (10 μg/ml), which binds to the HRR of HRG (Katagiri et al., 1987), partially inhibited the binding of HRG and HRG-IgG complexes in HRG$^P$ preparations (100 μg/ml) to necrotic Jurkat T cells by ~63% and ~67%, respectively (Fig. 3.18A, B and C). These data are consistent with earlier results that hemin can effectively block HRG binding to IgG$^HRG$ (see Section 3.3.1) as well as necrotic cell uptake enhanced by HRG$^P$ (see Section 3.3.7). Interestingly, the ability of hemin to block HRG binding to necrotic cells suggests that, besides the N1N2 domain of HRG (Jones et al., 2005b), the HRR of HRG may also mediate the binding of HRG to necrotic cells. In additional experiments, hemin was found to totally inhibit HRG$^{PID}$ binding to immobilized heparin (Fig. 3.18D), indicating that the interaction between HRG and hemin may modify the ligand binding capacity of HRG via a conformational change and/or directly masking the heparin binding sites on HRG. Therefore, hemin can potentially modify the ability of HRG to aid necrotic cell removal via a similar mechanism as heparin (see Section 3.3.8). However, the effect of hemin/haem on necrotic cell clearance enhanced by HRG in vivo following events, such as hemolysis (Hebbel and Eaton, 1989; Jacob, 1994), remains to be determined.
Figure 3.18. Hemin blocks the binding of HRG to necrotic cells and immobilized heparin.

(A) Effect of hemin (10 μg/ml) on the ability of HRG and HRG-IgG complexes in a HRG preparation (100 μg/ml) to bind to necrotic Jurkat T cells. Representative flow cytometry histograms are shown, with filled histograms representing the appropriate Ab only control and open blue and red histograms representing HRG and IgG binding in the absence or presence of hemin, respectively. HRG and IgG binding was detected by flow cytometry as described, respectively, in Figure 3.4 and 3.5. Quantitative comparison of (B) HRG and (C) IgG binding to necrotic cells based on the data from (A). (D) Effect of hemin on the binding of HRG-PID (2 μg/ml) to immobilized heparin was quantified by ELISA using wells pre-coated with 10 μg/ml of ExtrAvidin® and 10 μg/ml of biotinylated heparin. Error bars in each figure represent SEM (n = 3). ***, P < 0.001.
3.3.12 Necrotic cell ligand(s) of HRG

3.3.12.1 The role of DNA in HRG binding to necrotic cells

Studies by Gorgani et al. (2002) have previously suggested that HRG can bind directly to DNA and the exposure of DNA by late apoptotic cells mediates HRG binding. To investigate whether the binding of HRG and HRG-IgG complexes to necrotic cells is also mediated via DNA recognition, necrotic cells were treated with bovine DNase I to remove any exposed DNA. Similar to the experiment reported by Gorgani et al. (2002) using late apoptotic cells, there was >50% reduction in the binding of HRG and HRG-IgG complexes to DNase I (100 μg/ml) -treated necrotic cells (Fig. 3.19A and B). Although these results are consistent with previous studies by Gorgani et al. (2002), it is important to note that DNase I-treated necrotic cells not only showed a reduction in DNA content, as indicated by Hoechst 33258 staining, but also exhibited an unexpected decrease in cell size as indicated by a reduction in their FSC profile (Fig. 3.19C), suggesting a general loss of cellular components. To examine whether the cellular content of necrotic cells was being lost during DNase I treatment, necrotic cells were stained with either CFSE (cytoplasmic/nuclear protein stain) or PKH26 (membrane stain) to monitor, respectively, the integrity of cytoplasmic/nuclear proteins or the membrane content of necrotic cells. DNase I-treated necrotic cells showed a marked reduction in CFSE but not PKH26 staining (Fig. 3.19D), suggesting that the decrease in cell size following DNase I treatment could be the result of the cells losing their cytoplasmic and/or nuclear protein content. Since HRG binding to necrotic cells has been shown previously to be mediated through cytoplasmic ligand(s) (Jones et al., 2005b), the level of HRG binding to these cells is likely to be directly related to their cytoplasmic content and cell size. As shown is Figure 3.19E, higher levels of HRG bound to the larger necrotic cells, indicating that HRG binding is proportional to necrotic cell size. Although these data do not totally eliminate the possibility that HRG binds to necrotic cell DNA, the decrease in HRG and HRG-IgG complex binding to necrotic cells following DNase I treatment is likely to be the result of a general loss in cytoplasmic contents.
3.3.12.2 The role of cytoplasmic proteins in mediating HRG binding to necrotic cells

As described in Section 1.1, HRG has been shown to interact with a variety of different proteins including C1q, IgG, tropomyosin, plasminogen, fibrinogen and TSP (Jones et al., 2005a). Thus, the ability of HRG to bind to protein ligand(s) that are exposed in the cytoplasm of necrotic cells was investigated. Initially, pull-down experiments were performed using either HRG$^{\text{PID}}$- or BSA-conjugated Sepharose beads and cytoplasmic lysates isolated from the detergent-treated human melanoma MM170 cell line. Although the HRG$^{\text{PID}}$-beads bound to more cytoplasmic proteins compared to the control BSA-beads in pull-down experiments, no discreet protein bands were apparent (Fig 3.20). These results suggest that HRG may bind weakly and unspecifically to various cytoplasmic proteins.

3.3.12.3 HRG binds directly to cardiolipin

Recent studies by Ball-Rosen et al. (2007) have suggested that HRG may share functional similarities with another plasma protein, $\beta_2$GPI (also known as apolipoprotein H), an important phospholipid-binding protein that can aid the clearance of apoptotic cells (Balasubramanian and Schroit, 1998; Maiti et al., 2008). The authors demonstrated that patients with anti-phospholipid syndrome (APS), an autoimmune condition associated with high levels of autoantibodies against phospholipids/phospholipid-binding proteins/phospholipid-protein complexes such as the major autoantigen $\beta_2$GPI, possess autoantibodies that recognize HRG (Ball-Rosen et al., 2007). Although the interaction between HRG and the autoantibodies from APS patients was not thoroughly characterized by Ball-Rosen et al. (2007), numerous studies in the literature have demonstrated that the ability of autoantibodies from APS patients to recognize $\beta_2$GPI often, but not always, require the binding of $\beta_2$GPI to anionic phospholipids such as cardiolipin, which can induce the exposure of cryptic epitope(s) on $\beta_2$GPI (Valesini and Alessandri, 2005). Furthermore, since cardiolipin is normally located in the inner
Figure 3.19. Ability of HRG and HRG-IgG complexes to bind to DNA exposed on necrotic cells. Effect of DNase I pre-treatment (100 or 1000 µg/ml, 37°C, 90 min) on the ability of (A) HRG and (B) HRG-IgG complexes in a HRG preparation (100 µg/ml) to bind to necrotic Jurkat T cells. (C) Flow cytometry analysis of DNase I-treated (100 or 1000 µg/ml) necrotic cells for Hoechst 33258 staining to determine DNA content, and FSC to determine cell size. Representative flow cytometry plots are shown for necrotic cells treated with increasing concentrations of DNase I. (D) Effect of DNase I treatment on the ability of necrotic cells to retain CFSE and PKH26 staining. Viable Jurkat T cells were labeled with CFSE or PKH26 prior to the induction of necrosis (56°C, 30 mins) and DNase I treatment. Representative flow cytometry histograms are shown, with filled histograms representing the unstained necrotic cells only control and open blue, red and green histograms representing CFSE or PKH26 stained necrotic cells treated with 0, 100 or 1000 µg/ml of DNase I at 37°C for 90 mins, respectively. (E) Effect of cell size on the binding of HRG to necrotic cells. Necrotic cells were separated into small (R1), average (R2) and large (R3) cells according to FSC, with the corresponding MFI of HRG binding indicated in the right hand panel.
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A. HRG binding (Fold above background MFI) vs. DNase I treatment (μg/ml)

B. IgG binding (Fold above background MFI) vs. DNase I treatment (μg/ml)

C. Flow cytometry scatter plots showing Hoechst 33342 and FSC for different DNase I treatments.

D. Flow cytometry histograms showing CFSE and PKH26 fluorescence for different DNase I treatments.

E. Flow cytometry plots showing Hoechst 33342 and FSC for different cell sizes and gate MFI values:

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<td>Average</td>
<td>R2</td>
<td>61.7</td>
</tr>
<tr>
<td>Large</td>
<td>R3</td>
<td>78.1</td>
</tr>
</tbody>
</table>
Figure 3.20. HRG<sup>PID</sup> interacts with multiple cytoplasmic proteins. Ability of HRG<sup>PID</sup> to bind various cytoplasmic proteins as assessed by an affinity isolation procedure using HRG<sup>PID</sup>- or BSA-conjugated Sepharose beads and a cytoplasmic lysate isolated from detergent-treated MM170 cells (see Section 2.16). Following incubation of HRG<sup>PID</sup>- or BSA-conjugated beads (30 µl) with a cytoplasmic lysate (70 µl) at 4°C for 1 hr, beads were washed 3 times with PBS and beads-bound proteins were analyzed by SDS-PAGE, with HRG<sup>PID</sup>-beads or BSA-beads alone being included as negative controls and a cytoplasmic lysate (5 µl) as a positive control. Protein bands were detected by 0.2% Coomassie Brilliant Blue stain (protein stain).
mitochondrial membrane (McMillin and Dowhan, 2002), necrotic cells may expose intracellular phospholipids such as cardiolipin once the cell membrane and the mitochondrial membrane are permeabilized. Thus, the ability of HRG and HRG-IgG complexes to bind to phospholipids such as cardiolipin was investigated.

Initially, HRG$^\text{PID}$ was found to bind specifically to cardiolipin immobilized on ELISA wells (Fig. 3.21A), providing the first direct evidence that HRG can interact with a lipid ligand. Furthermore, HRG$^\text{PID}$ binding to cardiolipin and heparin was cross-inhibited by heparin (12.5 kDa) and cardiolipin, respectively (Fig. 3.21B and C). These results suggest that cardiolipin and heparin can either compete for the same/similar binding site on HRG (e.g. the HRR or the NIN2 domain) and/or a ligand-induced conformational change in HRG can affect both cardiolipin and heparin binding. To further examine the interaction between HRG and cardiolipin, viable Jurkat T cells were pre-coated with cardiolipin, a procedure that resulted in a marked increase in HRG$^\text{PID}$ binding to the viable Jurkat T cells (Fig. 3.21D), supporting the view that HRG can bind directly to cardiolipin. In order to assess whether endogenous cardiolipin can aid the binding of HRG to necrotic cells, doxorubicin (also known as adriamycin), a cytotoxic agent that binds cardiolipin and DNA (Mustonen and Kinnunen, 1993), was used as a blocking agent. Doxorubicin binding to necrotic cells was validated based on the fluorescence emission of doxorubicin at 575 nm (data not shown). Similar to previous studies that also used doxorubicin as an inhibitor to block C1q binding to cardiolipin on mitoplasts (isolated mitochondria that lack an outer membrane) (Peitsch et al., 1988), a relatively high concentration of doxorubicin (3000 μg/ml) but similar to that used by other groups (Peitsch et al., 1988), was required to partially inhibit (~30%) biotinylated HRG$^\text{PID}$ binding to necrotic cells (Fig. 3.21E). These data suggest that cardiolipin may function as an endogenous HRG ligand on necrotic cells. It is worth noting that the partial inhibitory effects of doxorubicin suggests either an inefficient blocking of cardiolipin recognition by HRG or additional ligand(s) are also involved in the binding of HRG to necrotic cells.
**Figure 3.21. HRG interacts with cardiolipin.** (A) Analysis of the ability of HRG<sup>PID</sup> to bind cardiolipin by ELISA using wells pre-coated with 50 μg/ml of cardiolipin, with ethanol coated wells being included as a negative control. (B) Effect of heparin (12.5 kDa) on the binding of HRG<sup>PID</sup> (5 μg/ml) to immobilized cardiolipin as quantified by ELISA using wells pre-coated with 50 μg/ml of cardiolipin. (C) Effect of soluble cardiolipin on HRG<sup>PID</sup> binding to immobilized heparin as detected by ELISA, with ELISA wells being pre-coated with 10 μg/ml of ExtrAvidin® and 10 μg/ml of biotinylated heparin, and then analyzed for HRG<sup>PID</sup> (1 μg/ml) binding in the presence of increasing concentrations of cardiolipin. (D) Ability of HRG in a HRG<sup>P</sup> preparation (100 μg/ml) to bind to viable Jurkat T cells pre-coated with cardiolipin (100 μg/ml, RT, 30 mins). HRG binding was detected by flow cytometry as described in Figure 3.4. Representative flow cytometry histograms are shown, with filled histograms representing appropriate Ab only control and open blue and red histograms representing HRG binding to untreated or cardiolipin coated cells, respectively. (E) Effect of doxorubicin (30-3000 μg/ml) on the ability of biotinylated HRG<sup>PID</sup> (100 μg/ml) to bind to necrotic Jurkat T cells. Biotinylated HRG<sup>PID</sup> binding was detected by flow cytometry using APC-conjugated streptavidin, with data being expressed as fold binding above background MFI. Error bars in each figure represent SEM (n = 3). NS, not significant. **, P < 0.01.
3.3.12.4 HRG binds to negatively charged lipids

To examine whether HRG can interact with other cellular lipids, lipid binding assays were performed using three commercially available membrane strips (Membrane Lipid Strips™, PIP Strips™ and SphingoStrips™) spotted with 100 pmoles of a variety of different biologically active lipids. Surprisingly, \( \text{HRG}^{\text{PID}} \) (1 µg/ml) bound strongly to phosphatidic acid (PA), sulfatide, phosphatidylinositol (PtdIns) (4)P, PtdIns(5)P and lysophosphatidic acid, as well as weakly to PtdIns(3,5)P \(_2\) and PtdIns(4,5)P \(_2\) (Fig. 3.22A, B and C). Except for sulfatide, which is a sulfated glycosphingolipids expressed on the cell surface (Ishizuka, 1997), the other lipids that showed interaction with \( \text{HRG}^{\text{PID}} \) are all intracellular lipids located on the inner leaflet of the cell membrane. Thus, these phospholipids could potentially act as endogenous ligand(s) for HRG in permeabilized necrotic and late apoptotic cells. In contrast, \( \text{HRG}^{\text{PID}} \) (1 µg/ml) did not bind to phospholipids that are usually exposed on early apoptotic cells, such as phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (Fig. 3.22A, B and C). These results are consistent with previous studies showing that HRG binds poorly to early apoptotic cells (Jones et al., 2005b). Contrary to the results shown in Figure 3.21 above, no binding of \( \text{HRG}^{\text{PID}} \) (1 µg/ml) was detected against cardiolipin spotted on the Membrane Lipid Strips™ (Fig. 3.22A), suggesting that the interaction between HRG and cardiolipin is substantially weaker compared to other phospholipids such as phosphatidic acid, sulfatide and PtdIns(4)P. It is worth noting that the binding conditions are considerably different between the lipid binding assay using lipid spotted membrane strips and the ELISA-based method described in Figure 3.21, with 100 pmoles of cardiolipin being spotted on the membrane strips, whereas a much higher concentration (~1950 pmoles) of cardiolipin was immobilized on the ELISA wells.

As described earlier, heparin can partially inhibit HRG binding to necrotic cells (see Section 3.3.8) as well as cardiolipin (see Fig. 3.21B). Therefore, the effect of heparin on the ability of HRG to bind to various phospholipids was initially examined using the PIP Strips™. The presence of heparin (10 µg/ml) had a partial inhibitory effect on \( \text{HRG}^{\text{PID}} \)
Figure 3.22. HRG<sup>PID</sup> interacts with multiple cellular lipids immobilized on membrane strips. Analysis of HRG<sup>PID</sup> (1 μg/ml) binding to an array of different cellular lipids using (A) Membrane Lipid Strips<sup>TM</sup>, (B) PIP Strips<sup>TM</sup> and (C) SphingoStrips<sup>TM</sup>, which are commercially available membrane strips immobilized with 100 pmoles of the indicated biologically active lipids, with HRG binding being detected by a mouse anti-human HRG mAb (HRG-4) and a HRP-conjugated sheep anti-mouse Ig Ab. Lipids to which HRG exhibited strong or weak binding are shown in red and blue text, respectively.
(1 µg/ml) binding to various phospholipids (Fig. 3.23A), suggesting that the heparin binding domain on HRG and/or a heparin-induced conformational change in HRG is important for phospholipid binding. Similarly, the presence of cardiolipin (50 µg/ml) also had a partial but less substantial inhibitory effect on HRG<sup>PID</sup> (1 µg/ml) binding to various phospholipids (Fig. 3.23B). It is worth noting that there are some differential inhibitory effects of heparin and cardiolipin on HRG<sup>PID</sup> binding to phospholipids spotted on the PIP Strips<sup>TM</sup>, whereby some phospholipids such as lysophosphatidic acid are more sensitive to inhibition by heparin than cardiolipin, and vice versa for PtdIns(4)P, possibly due to the different concentrations of heparin and cardiolipin used in the experiment, their relative binding affinity for HRG and the region of HRG they bind to.

Besides the membrane strip-based lipid binding assay, the ability of HRG to interact with various phospholipids was also validated via ELISA. Similar to the results above, HRG<sup>PID</sup> bound directly to phospholipids immobilized on ELISA plates, in particular sulfatide and PtdIns(4)P (Fig. 3.24A). In contrast to the membrane strip-based lipid binding assay, a direct interaction of HRG<sup>PID</sup> with PtdIns and cardiolipin was also detected via ELISA (Fig. 3.24A), possibly due to a higher concentration of HRG<sup>PID</sup> and phospholipids being used in the ELISA-based assay. These results suggest that at physiological concentrations of HRG (i.e. ~100-150 µg/ml), HRG is likely to interact with various abundant phospholipids. In additional experiments, the presence of heparin and hemin markedly inhibited HRG<sup>PID</sup> binding to all the lipids tested (Fig. 3.24B), suggesting that the heparin and hemin binding domain on HRG and/or a ligand-induced conformational change is important for the interaction of HRG with phospholipids. Importantly, HRG-IgG complexes (containing approximately a 1:1 molar ratio of HRG<sup>PID</sup>:IgG<sup>H<sub>H</sub>RG</sup>, 4 µg/ml), but not IgG<sup>H<sub>H</sub>RG</sup> alone or the human IgG2κ myeloma protein (4 µg/ml), bound strongly to various phospholipids, in particular sulfatide, PA and PtdIns(4)P (Fig. 3.24C). Collectively, these data suggest that HRG and HRG-IgG complexes can bind specifically to various phospholipids. Thus, HRG<sup>P</sup>-mediated necrotic cell clearance may involve the binding of HRG and HRG-IgG complexes to a variety of phospholipids that are exposed in necrotic cells but not by apoptotic or viable cells.
Figure 3.23. Interaction between HRC<sup>PID</sup> and cellular lipids is partially inhibited by heparin and cardiolipin. Effect of (A) heparin (10 µg/ml) and (B) cardiolipin (50 µg/ml) on HRC<sup>PID</sup> (1 µg/ml) binding to the different HRG-binding lipids on PIP Strips<sup>TM</sup>, with densitometry analysis of HRG binding to the different immobilized lipids shown in the right hand panels. For the identity of the different lipid spots on the PIP Strips<sup>TM</sup> see Figure 3.22B.
Figure 3.24. Binding of HRG\textsuperscript{PID} and HRG-IgG complexes to different cellular lipids immobilized on ELISA plates. (A) Analysis of the ability of HRG\textsuperscript{PID} to bind various phospholipids by ELISA using wells pre-coated with 10 \mu g/ml of cardiolipin, sulfatide, phosphatidic acid (PA), phosphatidylinositol (PtdIns), PtdIns(4)P and PtdIns(4,5)P\textsubscript{2}, with ethanol (EtOH) coated wells being included as a negative control. (B) Effect of heparin (12.5 kDa) and hemin on HRG\textsuperscript{PID} binding to various phospholipids was also examined by ELISA using wells pre-coated with different phospholipids as described in (A). (C) Analysis of the ability of HRG-IgG complexes (approximately 1:1 molar ratio of HRG\textsuperscript{PID}:IgG\textsuperscript{HRG}, 4 \mu g/ml) to bind to various phospholipids as detected by ELISA using wells pre-coated with different phospholipids as described in (A), with IgG\textsuperscript{HRG} (4 \mu g/ml) or a human IgG2\textsubscript{x} myeloma (4 \mu g/ml) being included as negative controls.
To examine whether phospholipids such as cardiolipin, PA, PtdIns(4)P and PtdIns(5)P are the endogenous necrotic cell ligand(s) for HRG, necrotic cells were treated with *Bacillus cereus* phospholipase C (PLC) which removes the phosphate headgroup of various phospholipids. As a positive control to validate PLC activity, annexin-V binding to PS exposed in necrotic cells was completely abolished by PLC treatment (Fig. 3.25A). Unexpectedly, PLC treatment potentiated the binding of HRG and HRG-IgG complexes in HRG preparations (100 µg/ml) to necrotic cells (Fig. 3.25B). Besides affecting annexin-V and HRG binding, PLC treated necrotic cells also exhibited an increase in cellular granularity as indicated by their SSC profile (Fig. 3.25C). Since an increase in cellular granularity is a feature that correlates with necrosis (Vanden Berghe *et al.*, 2004), these data suggest that the exposure of necrotic cells to PLC may alter the cellular architecture of the necrotic cells and expose more HRG binding sites. Furthermore, since PLC can effectively hydrolyze PS exposed in necrotic cells (Fig. 3.25A), the failure of PLC treatment to reduce HRG binding to necrotic cells (Fig. 3.25B) further confirms earlier binding studies (Fig. 3.22A and B) and is consistent with previous studies by Gorgani *et al.* (2002), showing that PS is unlikely to function as an endogenous permeabilized/necrotic cell ligand for HRG. It is also worth noting that *Bacillus cereus* PLC preferentially hydrolyzes phospholipids such as PE and phosphatidylglycerol (PG) more efficiently than cardiolipin (Mavis *et al.*, 1972). Therefore, due to potential technical difficulties, it cannot be established whether endogenous phospholipids exposed in necrotic cells represent major HRG ligand(s).

### 3.3.12.5 Phosphorylated proteins may also represent an HRG ligand in necrotic cells

It has been reported previously that intracellular signalling molecules such as Shc can recognize both phospholipids and tyrosine-phosphorylated proteins via the phosphotyrosine binding (PTB) domain to facilitate its localization to the cell membrane and to propagate signal transduction events (Zhou *et al.*, 1995; Ravichandran *et al.*, 1997). Most strikingly, similar to HRG as described in Section 3.3.12.4 above, PTB
Figure 3.25. Ability of HRG and HRG-IgG complexes to bind to phospholipase C-treated necrotic cells. (A) Effect of Bacillus cereus phospholipase C (PLC) treatment (2 unit/ml, 37°C, 90 mins) of necrotic cells in annexin-V-FITC binding, with viable cells being included as a negative control. Representative flow cytometry histograms are shown, with filled histograms representing unstained control and open red and blue histograms representing annexin-V-FITC binding to untreated or PLC-treated cells, respectively. (B) Ability of HRG and HRG-IgG complexes in a HRG preparation (100 μg/ml) to bind to untreated or PLC-treated (2 unit/ml, 37°C, 90 mins) necrotic cells. HRG and IgG binding was analyzed by flow cytometry as described in Figure 3.4 and 3.5, respectively. Representative flow cytometry histograms are shown, with filled histograms representing appropriate Ab only control, and open red and blue histograms representing HRG/IgG binding to untreated or PLC-treated necrotic cells, respectively. (C) Analysis by flow cytometry of the FSC and SSC profiles of necrotic cells treated with PLC (2 unit/ml) at 37°C for 90 mins. Representative flow cytometry dot plots and histograms are shown, with open red and blue dot plots/histograms representing untreated or PLC-treated necrotic cells, respectively.
domain containing proteins such as Shc and disabled 1 (Dab1) were found to bind more efficiently to PtdIns(4)P and PtdIns(4,5)P2 than other negatively charged lipids like PtdIns, PS or PE (Zhou et al., 1995; Howell et al., 1999). Therefore, HRG may have similar ligand binding properties as intracellular signalling proteins containing the PTB domain and can potentially interact with phosphorylated proteins exposed in permeabilized/necrotic cells. Initial experiments were performed using ELISA to determine the ability of HRG to bind to phosphoserine-, phosphotyrosine- or phosphothreonine-conjugated BSA. Surprisingly, HRG^{PID} and HRG-IgG complexes (containing approximately a 1:1 molar ratio of HRG^{PID}, IgG^{HRG}, 4 μg/ml) bound more avidly to wells pre-coated with BSA-phosphoserine than those pre-coated with BSA-phosphotyrosine or BSA-phosphothreonine (Fig. 3.26A and B). Similar to earlier results where heparin and hemin can effectively block HRG binding to necrotic cells and various phospholipids (see Section 3.3.8, 3.3.10, 3.3.11, 3.3.12.3 and 3.3.12.4), heparin and hemin also markedly inhibited HRG^{PID} binding to BSA-phosphoserine (Fig. 3.26C). In additional experiments, BSA-phosphoserine was found to partially inhibit HRG^{PID} (25 μg/ml) binding to necrotic cells (Fig. 3.26D). Collectively, these data suggest that phosphorylated proteins, in particular those containing phosphoserine, may function as necrotic cell ligand(s) for HRG. Consistent with this view, phosphoserine/threonine was exposed predominately on necrotic but not viable cells (Fig. 3.26E). To further examine whether phosphorylated proteins are the endogenous necrotic cell ligand(s) for HRG, necrotic cells were treated with bovine alkaline phosphatase (AP) which dephosphorylates various proteins. As a positive control to validate AP activity, the exposure of phosphoserine/threonine on necrotic cells, as detected by an anti-phosphoserine/threonine mAb (22A), was reduced by >70% following AP treatment (Fig. 3.26F). However, AP treatment showed no apparent effect on HRG^{PID} (25 μg/ml) binding to necrotic cells (Fig. 3.26G), indicating that the exposure of serine/threonine-phosphorylated proteins at a level of ~20-25% on necrotic cells is sufficient to aid HRG binding and/or other endogenous necrotic cell ligand(s) may compensate for the loss of phosphorylated proteins and mediate HRG binding. Interestingly, it is worth noting that besides recognizing tyrosine-phosphorylated proteins or phospholipids, PTB domains from X11, Numb and Dab1 can also bind specifically to non-phosphorylated peptides
Thus, the similarities shared between HRG and other PTB domain containing proteins such as Dab1, in respect to ligand binding, suggests that HRG can potentially recognize specific protein sequences either in the presence or absence of phosphorylation. This may account for the inability of AP treatment to reduce HRG binding to necrotic cells.

3.3.13 Phagocytosis of necrotic cells via HRG\(^p\) promotes a pro-inflammatory response

As discussed in Section 1.2, necrotic cell removal is often associated with a pro-inflammatory response, possibly due to the exposure of immunostimulatory molecules known as damage-associated molecular patterns (DAMPs) to the immune system when cells become permeabilized/necrotic (Kono and Rock, 2008). Furthermore, the cross-linking of activating Fc\(\gamma\)R, such as human Fc\(\gamma\)RI and Fc\(\gamma\)RIIA, which initiates downstream signalling events via the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) (Strzelecka et al., 1997), is also known to induce the production and secretion of pro-inflammatory cytokines such as IL-6 (Krutmann et al., 1990), TNF (Debets et al., 1990) and IL-8 (Marsh et al., 1995; Marsh et al., 1996). Therefore, the ability of THP-1 cells to release a panel of different pro-inflammatory cytokines following necrotic cell uptake, either in the presence or absence of HRG\(^p\), was investigated using a human inflammatory cytokine bead array (CBA) assay (see Section 2.21 for details). As a positive control, THP-1 cells were treated with a range of concentrations of LPS (1.25-10 \(\mu\)g/ml) for 6 hrs, LPS being found to induce the release of IL-8, TNF as well as a small amount of IL-6 into the culture supernatants (Fig. 3.27A). Stimulation of THP-1 cells with 10 \(\mu\)g/ml of LPS for 24 hrs also induced the secretion of IL-1\(\beta\) but not IL-10 or IL-12p70 (data not shown). These results demonstrate that LPS can effectively induce the production of pro-inflammatory cytokines such as IL-8, TNF, IL-6 and IL-1\(\beta\), but not IL-10 or IL-12p70, from THP-1 cells. To ensure that the effects of HRG\(^p\) on the production cytokines by THP-1 cells is not due to the presence of contaminating LPS in the HRG\(^p\) preparations (~70 ng of LPS
Figure 3.26. Binding of HRG to phosphorylated amino acid residues. (A) Analysis of the ability of HRG^PID to bind phosphoserine-, phosphotyrosine- and phosphothreonine-conjugated BSA by ELISA using wells pre-coated with 10 μg/ml of BSA-phosphoserine, BSA-phosphotyrosine or BSA-phosphothreonine, with BSA coated wells being included as a negative control (subtracted as background binding). (B) Analysis of the ability of HRG-IgG complexes (approximately 1:1 molar ratio of HRG^PID:IgG^HRG, 4 μg/ml) to bind BSA-phosphoserine, BSA-phosphotyrosine and BSA-phosphothreonine by ELISA as described in (A). (C) Effect of heparin (12.5 kDa) and hemin on HRG^PID (5 μg/ml) binding to BSA-phosphoserine was also examined by ELISA using wells pre-coated with 10 μg/ml of BSA-phosphoserine. (D) Effect of BSA-phosphoserine (400 μg/ml) on the ability of HRG^PID (25 μg/ml) to bind to necrotic Jurkat T cells, with BSA (400 μg/ml) being included as a negative control. (E) Analysis of the exposure of phosphoserine/threonine residues on viable and necrotic cells, with phosphoserine/threonine being detected by flow cytometry using a mouse anti-phosphoserine/threonine mAb (22A) and a PE-conjugated sheep F(ab')2 anti-mouse Ig Ab. Representative flow cytometry histograms are shown, with filled histograms representing isotype and secondary Ab only control, and open black histograms representing mouse anti-phosphoserine/threonine mAb (22A) binding. (F) Effect of bovine alkaline phosphatase (AP) treatment (250 unit/ml, 37°C, 60 mins) of necrotic cells in the exposure of phosphoserine/threonine residues as detected by flow cytometry as described in (E), with untreated necrotic cells being included as a negative control. (G) Ability of HRG^PID (25 μg/ml) to bind to untreated or AP-treated (250 unit/ml, 37°C, 60 mins) necrotic cells. HRG binding was analyzed by flow cytometry as described in Figure 3.4. Representative flow cytometry histograms are shown, with filled histograms representing appropriate Ab only control, and open red and blue histograms representing HRG binding to untreated or AP-treated necrotic cells, respectively. Error bars in (A), (C) and (D) represents SEM (n = 3). *** P < 0.001.
Figure 3.27. Pro-inflammatory cytokines are released following HRG\textsuperscript{P}-mediated uptake of necrotic cells. (A) Production of pro-inflammatory cytokines by THP-1 cells stimulated for 6 hrs at 37°C with increasing concentrations of LPS (1.25-10 \(\mu\)g/ml), with cytokine production being assessed by a cytokine bead array (CBA) assay. (B) Ability of endotoxin-free HRG\textsuperscript{P} (150 \(\mu\)g/ml) to enhance the phagocytosis of CFSE positive necrotic Jurkat T cells by PKH26 positive THP-1 cells as described in Figure 3.7. Representative flow cytometry plots are shown for phagocytic assays performed in the presence or absence of endotoxin-free HRG\textsuperscript{P}, with gated regions being PKH26 positive THP-1 cells that have or have not phagocytosed CFSE positive necrotic cells. Values in each gated area represent percentage of cells in the assay. (C-H) Production of pro-inflammatory cytokines IL-8 (C), TNF (D), IL-10 (E), IL-12p70 (F), IL-1\(\beta\) (G) and IL-6 (H) by THP-1 cells following incubation for 2-12 hrs at 37°C with necrotic Jurkat T cells (10:1 necrotic Jurkat:THP-1 ratio), either in the presence or absence of endotoxin-free HRG\textsuperscript{P} (150 \(\mu\)g/ml). Cytokine levels as determined by a CBA assay, with THP-1 cells alone or in the presence of endotoxin-free HRG\textsuperscript{P} being included as negative controls. Error bars represent the range of duplicate samples. 5 pg/ml represent the limit of detection by the CBA assay.
Chapter 3

A 6 hrs

Cells only
Endotoxin-free HRGP

B

Cells only
Endotoxin-free HRGP

C

IL-8 (pg/ml)

D

TNF (pg/ml)

E

IL-10 (pg/ml)

F

IL-12/70 (pg/ml)

G

IL-1β (pg/ml)

H

IL-6 (pg/ml)

- + + + THP-1 cells
- + + - Necrotic cells
- - - - Endotoxin-free HRGP

- + + + THP-1 cells
- + - - Necrotic cells
- - - - Endotoxin-free HRGP

- + + + THP-1 cells
- + - - Necrotic cells
- - - - Endotoxin-free HRGP

- + + + THP-1 cells
- + - - Necrotic cells
- - - - Endotoxin-free HRGP
per 1 mg of HRG\(^p\), data not shown), LPS was removed from the HRG\(^p\) preparations using an EndoTrap\(^\circledR\) column (see Section 2.4 for details). As shown in Figure 3.27B, endotoxin-free HRG\(^p\) (150 \(\mu\)g/ml) effectively enhanced the phagocytosis of necrotic cells and this preparation of HRG\(^p\) was used in all subsequent cytokine production assays.

Necrotic cells alone induced THP-1 cells to produce IL-8, but no other cytokine tested (Fig. 3.27C to H), which could be the result of the exposure of endogenous DAMPs such as HMGB1 by the necrotic cells (Andersson et al., 2000). In contrast, necrotic cells in the presence of endotoxin-free HRG\(^p\) (150 \(\mu\)g/ml) resulted in enhanced synthesis of both IL-8 and TNF by THP-1 cells (Fig. 3.27C and D), suggesting that the enhanced necrotic cell disposal induced by HRG\(^p\) can promote the release of certain pro-inflammatory cytokines. It is worth noting that in the absence of necrotic cells, endotoxin-free HRG\(^p\) (150 \(\mu\)g/ml) also induced the release of very small quantities of IL-8 (Fig. 3.27C), possibly caused by the direct activation of Fc\(\gamma\)R on THP-1 cells by HRG-IgG immune complexes. Furthermore, no apparent induction of IL-10, IL-12p70, IL-1\(\beta\) or IL-6 was observed under all conditions tested (Fig. 3.27E to H), suggesting that necrotic cell clearance, either in the absence or presence of HRG\(^p\), does not modulate the production of these cytokines by the monocytic cell line THP-1. Collectively, these data suggest that HRG\(^p\)-mediated necrotic cell uptake can enhance the release of pro-inflammatory cytokines such as IL-8 and TNF by THP-1 cells and facilitate the induction of a necrotic cell-dependent inflammatory response.

3.4 Discussion

Despite a growing number of opsonins being identified as key adaptor molecules in aiding apoptotic and necrotic cell clearance, the molecular mechanisms (i.e. the phagocytic receptors, dying/dead cell ligands, and immunological consequences) underpinning these phagocytic pathways are often poorly characterized (see Section 1.2). The results presented in this chapter demonstrate that the abundant plasma glycoprotein, HRG, can form a stable complex with IgG and tether a specific subclass of
IgG to necrotic but not viable cells via negatively charged molecules such as phospholipids and possibly phosphorylated proteins exposed in necrotic cells. Furthermore, HRG-IgG opsonized necrotic cells are specifically recognized by FcγR and cell surface HS on phagocytes, subsequently triggering the phagocytic uptake of necrotic cells and initiating a necrotic cell-dependent inflammatory response (summarized in Fig. 3.28). Thus, recognition of necrotic cells via a HRG-mediated pathway may play an important role in mediating the recruitment of other leukocytes such as neutrophils and macrophages to sites of tissue injury.

Initially, IgG was found to co-purify with human HRG$^P$ (Fig. 3.1). These data are consistent with previous studies showing that HRG purified from the human plasma may contain trace amounts of various HRG ligands including IgG (Leung et al., 1983). In contrast, detectable levels of C1q (Fig. 3.11A) or plasminogen (data not shown) were not found in the HRG$^P$ preparations. Interestingly, there was a skewed distribution of IgG subclasses (i.e. IgG2κ) co-purifying with HRG compared to normal human plasma (Fig. 3.2). These data were consistent with previous studies showing that HRG binds preferentially to human IgG1κ and IgG2κ myeloma proteins (Gorgani et al., 1999c). It should be noted, however, that HRG$^{PID}$ bound more avidly to IgG$^{HRG}$ than IgG1κ and IgG2κ myeloma proteins (Fig. 3.3A), suggesting that the interaction between HRG and the co-purified IgG may depend on other factors besides the IgG subclass. Since many plasma proteins such as C1q, CRP, SAP, MBL and β$_2$GPI are targeted by autoantibodies in patients with autoimmune diseases such as SLE (Limburg and Bijl, 2005; Shoenfeld et al., 2007) and APS (Shoenfeld et al., 2008), the co-purified IgG in the HRG$^P$ preparations may represent high affinity anti-HRG autoantibodies. Consistent with this view, IgG from APS patients was recently shown to bind to both the major autoantigen β$_2$GPI as well as HRG (Ball-Rosen et al., 2007). In addition, anti-β$_2$GPI autoantibodies in APS patients were also found to be significantly skewed towards the IgG2 subclass (Guerin et al., 1999). However, the ability of HRG to bind directly to IgG itself (Gorgani et al., 1997; Gorgani et al., 1999c) poses potential technical difficulties in confirming whether the interaction between HRG and the co-purified IgG is truly mediated via the
Figure 3.28. Proposed molecular mechanism of HRG<sup>P</sup>-mediated phagocytosis of necrotic cells by monocytes. (1) HRG tethers human IgG (in particular IgG2k) to necrotic cells and enhances phagocytic uptake of necrotic cells by monocytes via a FcγR-dependent mechanism. (2) In contrast, HRG binds viable cells via cell surface heparan sulfate proteoglycans (HSPG), which prevents HRG interacting with human IgG and thus prevents the phagocytosis of viable cells. HSPG on monocytes also aids the uptake of necrotic cells either (3) through direct interaction of HSPG with necrotic cells or (4) indirectly via cell surface-bound HRG interacting with necrotic cells. (5) HRG can potentially bind cellular lipids and phosphorylated proteins exposed on necrotic cells, possibly via the N1N2 domain and/or the HRR of HRG. (6) HRG<sup>P</sup>-mediated uptake of necrotic cells induces the release of pro-inflammatory cytokines such as TNF and IL-8.
antigen binding properties of the co-purified IgG. Nevertheless, pooled normal human IgG from 8-15 healthy individuals (Sigma-Aldrich, St. Louis, MO) was found to bind more avidly to immobilized HRG compared to other human IgG myeloma proteins (data not shown), suggesting that autoantibodies or even ‘natural’ antibodies may exist in clinically normal individuals.

Although the co-purified IgG constituted only approximately 1-4% of the HRG\textsuperscript{p} preparations (Fig. 3.1E), the co-purified IgG was essential for HRG\textsuperscript{p}-mediated disposal of necrotic cells (Fig. 3.7C and D). The ability of HRG to tether the co-purified IgG specifically to permeabilized/necrotic but not viable cells (Fig. 3.5 and 3.6) provided an important ‘eat-me’ signal on necrotic cells to trigger phagocytic uptake by THP-1 cells (Fig. 3.7C and D). These results are consistent with previous studies showing that HRG\textsuperscript{p} selectively enhances the clearance of necrotic but not viable or early apoptotic cells (Jones \textit{et al.}, 2005b). It is also important to note that viable cells may also play an active role in preventing the binding of HRG-IgG complexes to the cell surface, as the presence of HS, a key cell surface ligand for HRG (Jones \textit{et al.}, 2004b), inhibited the binding of HRG to IgG\textsuperscript{HRG} (Fig. 3.13B). Thus, although HRG was able to bind to the cell surface of viable cells (Fig. 3.4C to E), the binding of HRG to cell surface HS is likely to prevent HRG from interacting with the co-purified IgG. In contrast, HRG-IgG complexes bound avidly to various intracellular phospholipids such as PtdIns(4)P and cardiolipin (Fig. 3.24C), suggesting that the exposure of intracellular phospholipids when the cell membrane becomes permeabilized may aid the binding of HRG-IgG complexes to necrotic cells and enhance necrotic cell removal. Furthermore, the data presented in this chapter also demonstrate that the level of HRG binding to necrotic cells is not an important factor in initiating phagocytic uptake, as HRG in both the HRG\textsuperscript{p} and HRG\textsuperscript{PID} preparations bound to a similar extent to necrotic cells (Fig. 3.4C and D) but only HRG\textsuperscript{p} was able to efficiently enhance necrotic cell uptake due to the binding of HRG-IgG complexes to necrotic cells (Fig. 3.4 to 3.7).

It has been reported frequently in the literature that the purity of proteins used in various \textit{in vitro} and \textit{in vivo} assays is crucial to accurately determine their biological functions,
and possibly the cause of conflicting data obtained by different laboratories. For example, the suggestion that human CRP directly interacts with FcγR has been challenged by the discovery that IgG is present in CRP purified from human pleural fluid (Hundt et al., 2001). The ability of recombinant human heat shock proteins 60 and 70 to induce the production of TNFα by macrophages has also been questioned due to the presence of endotoxin in the recombinant protein preparations (Gao and Tsan, 2003a; Gao and Tsan, 2003b). Therefore, the presence of IgG in the HRG preparations purified from the human plasma may cast doubts on the conclusion made by previous studies, especially those related to FcγR function. In a recent study by Gorgani et al. (2002), HRG was proposed to function as a bridging molecule that directly interacts with both late apoptotic cells and FcγRI on phagocytes. However, in contrast to Gorgani et al. (1999b) and Gorgani et al. (2002), the experimental data presented in this study demonstrate that HRG is unable to bind directly to FcγR (Fig. 3.10B and E). Instead, the co-purified IgG in the HRG preparations was found to be the key component in binding to FcγRI on THP-1 cells (Fig. 3.10C and D). Since HRG-IgG complexes were also found to tether specifically to permeabilized late apoptotic and secondary necrotic cells (Fig. 3.6A), it is plausible that the ability of HRG to aid the clearance of late apoptotic cells, as reported by Gorgani et al. (2002), is mediated via the co-operative effect of HRG and the co-purified IgG, but not HRG alone.

Although FcγRI was found to be the key receptor on THP-1 cells that mediated the clearance of necrotic cells opsonized by HRG-IgG complexes (Fig. 3.8B and C), the removal of cell surface HS on THP-1 cells by human heparanase treatment also resulted in a marked reduction in HRG binding to THP-1 cells (Fig. 3.16B), as well as HRG-mediated uptake of necrotic cells (Fig. 3.16C and D). Thus, FcγRI and cell surface HS on phagocytes may function co-operatively in recognizing and removing necrotic cells via a HRG-dependent mechanism. Whilst previous studies by Gebska et al. (2002) have suggested that cell surface HS on phagocytes can aid the disposal of dying cells, the results presented in this study provide the first direct evidence that cell surface HS on phagocytes is indeed important for necrotic cell clearance and involves heparin/HS-binding opsonins such as HRG. Furthermore, it is worth noting that, since HRG has been
suggested to contain two heparin/HS binding domains located in the HRR (Borza et al., 1996; Borza and Morgan, 1998; Vanwildeemeersch et al., 2006) and the N-terminal domain of HRG (Brown and Parish, 1994; Jones et al., 2004b), HRG can potentially function as a bridging molecule between cell surface HS on both phagocytes and viable cells. However, HRG\textsuperscript{PID} bound to cell surface HS was unable to enhance biotinylated heparin binding to viable cells (data not shown), indicating that HRG is unlikely to act as an adaptor protein between two HS chains and thus cannot enhance viable cell uptake by phagocytes (Jones et al., 2005b).

In order to trigger phagocytic uptake, dying cells must expose a specific array of ‘eat-me’ and ‘don’t eat-me’ signals, which can be detected by phagocytes via a range of receptors and opsonins (Grimsley and Ravichandran, 2003). The experimental data presented in this chapter demonstrate that the ‘eat-me’ signal(s)/necrotic cell ligand(s) recognized by HRG are present ubiquitously in permeabilized cells and processes such as apoptosis or heat induced cell death are not required to generate the ligand(s) (Fig. 3.6). Furthermore, HRG and HRG-IgG complexes were also found to bind to heat induced necrotic cells generated from human T cells (Jurkat) (Fig. 3.4 and 3.5), Chinese hamster ovary cells (pgsA-745) (Fig. 3.17E and F) and mouse thymoma cells (EL4) (data not shown), indicating that most cell types are likely to express the necrotic cell ligand(s) of HRG. Although pull-down experiments using HRG\textsuperscript{PID}-beads and cytoplasmic lysates did not identify any proteins that can specifically interact with HRG (Fig. 3.20), anionic phospholipids (Fig. 3.21 to 3.24) and phosphorylated proteins (Fig. 3.26) were demonstrated as potential necrotic cell ligand(s) of HRG, which are molecules that can be found in most mammalian cells and are often shielded from the extracellular environment unless the cell membrane has become permeabilized. Unexpectedly, HRG was found to bind avidly to intracellular phosphoinositide signalling molecules such as PtdIns(4)P, PtdIns(5)P and PtdIns(4,5)P\textsubscript{2} but not PtdIns(3,4)P\textsubscript{2} or PtdIns(3,4,5)P\textsubscript{3} (Fig. 3.22A and B). The ability of HRG to bind to various phospholipids with such striking specificity demonstrates that HRG does not simply interact with highly charged anionic molecules, but recognizes specific molecular structures. Interestingly, besides intracellular molecules such as γ-adaptin and ceramide-
transfer protein (D'Angelo et al., 2008), serum proteins like rat MBL (Kuroki et al., 1997) and human HRG (Fig. 3.22 and 3.24) are the only two extracellular proteins identified to date that can bind specifically to PtdIns(4)P, which is the most abundant form of the monophosphorylated inositol phospholipids in mammalian cells (D'Angelo et al., 2008). Although the physiological significance of MBL binding to PtdIns(4)P has not been elucidated, the results presented in this study suggest that intracellular phospholipids like PtdIns(4)P may function as permeabilized/necrotic cell-specific 'eat-me' signal or DAMPs, which can be recognized by phagocytes via serum opsonins such as HRG and possibly MBL. Indeed, MBL has been shown recently to bind strongly to late apoptotic and necrotic cells but not early apoptotic cells (Nauta et al., 2003). Furthermore, since the binding of β2GPI to apoptotic cells via anionic phospholipids has been proposed to initiate the break of tolerance and induce the generation of anti-β2GPI or anti-phospholipid-β2GPI complex autoantibodies (Levine et al., 1998), the interaction between HRG and phospholipids such as cardiolipin in necrotic cells (Fig. 3.21) may provide a potential mechanism for generating anti-HRG autoantibodies as proposed by Ball-Rosen et al. (2007).

In addition to the results presented in this chapter, several previous studies have also reported the ability of antibodies to bind specifically to dying/dead cells, but not viable cells (see Section 1.2.3.2). For example, 'natural' IgM antibodies were found to bind to structures such as lysophosphatidylcholine, oxidized LDL and phosphorylcholine exposed on apoptotic cells (Shaw et al., 2000; Kim et al., 2002), which may play an important role in the clearance of apoptotic cells via the activation of the classical complement pathway (Kim et al., 2002; Ogden et al., 2005; Quartier et al., 2005). Similarly, normal plasma IgG as well as IgG autoantibodies from SLE patients were also found to bind predominately to permeabilized/necrotic cells (Ciurana et al., 2004; Zwart et al., 2004; Grossmayer et al., 2007; Reefman et al., 2007). In contrast, IgG autoantibodies that can recognize either the β2GPI or phospholipid-β2GPI complex in patients with autoimmune diseases were found to bind specifically to apoptotic but not viable cells via a β2GPI-dependent mechanism (Manfredi et al., 1998b). Although it has been reported that β2GPI itself can aid the uptake of apoptotic cells by acting as an
adaptor molecule between PS on apoptotic cells and lipoprotein receptor-related protein on phagocytes (Maiti et al., 2008), the presence of IgG autoantibodies against β2GPI or the phospholipid-β2GPI complex was shown to markedly enhance the uptake of apoptotic cells and induce the production of pro-inflammatory cytokines such as TNFα (Manfredi et al., 1998a). Furthermore, targeting β2GPI opsonized apoptotic cells to dendritic cells via a rabbit anti-β2GPI Ab has also been demonstrated to enhance the immunogenicity of apoptotic cells (Rovere et al., 1999). Thus, HRG-IgG complexes may function via the same mechanism as β2GPI/anti-β2GPI autoantibodies in facilitating the disposal of dying/dead cells by acting as a bridging complex between phospholipids exposed in necrotic cells and FcyR on phagocytes. Similarly, opsonization of necrotic cells by HRG-IgG complexes also enhanced the production of pro-inflammatory cytokines such as IL-8 and TNF by THP-1 cells (Fig. 3.27C and D), possibly via the activation of FcyR. Therefore, the results presented in this chapter provide vital clues to understanding the molecular mechanisms and immunological consequence underpinning the clearance of permeabilized/necrotic cells via HRG, which may have important implications in the development and maintenance of autoimmune diseases such as SLE and APS.
4.1 Abstract

The plasminogen/plasmin system is involved in a broad range of normal physiological and pathological processes, such as tissue remodelling, angiogenesis, wound healing, embryogenesis and tumour metastasis. Plasminogen activators and receptors for plasminogen/plasminogen activators are essential for the processing of plasminogen to the active serine protease, plasmin. Plasmin can in turn positively or negatively regulate further plasminogen activation via plasmin-mediated cleavage of receptors and activators. Histidine-rich glycoprotein (HRG) is a 75 kDa glycoprotein found in human plasma at the relatively high concentration of ~100-150 µg/ml. HRG has a multi-domain structure that can interact with a variety of molecules such as Zn$^{2+}$, heparin, heparan sulfate (HS), IgG, fibrinogen and plasminogen. Previous studies have reported that HRG can act as an adaptor molecule to tether plasminogen with high affinity to glycosaminoglycan (GAG) bearing surfaces and regulate plasminogen activation via a
number of different mechanisms. Since HRG itself is sensitive to plasmin cleavage, this chapter describes experiments that examine in detail the cleavage of human HRG by plasmin and the effect of this cleavage on various functions of HRG. Importantly, most of the HRG fragments generated by plasmin cleavage are held together by disulfide linkages and, therefore, are not released from the molecule under non-reducing conditions. It was found that plasmin-mediated cleavage partially inhibits HRG binding to cell surface HS, but enhances HRG binding to necrotic cells and to plasminogen. However, both intact and plasmin-cleaved HRG enhanced the binding of plasminogen to heparin-coated surfaces to a similar extent. Furthermore, the presence of heparin, Zn$^{2+}$ or acidic pH was found to protect HRG from plasmin cleavage. Based on these data, proteolytic cleavage of HRG by plasmin may provide a feedback mechanism to regulate the effects of HRG on the plasminogen/plasmin system as well as other functions of HRG.

4.2 Introduction

Serum protein-based biological systems such as the complement, coagulation and fibrinolytic pathways often rely heavily on the proteolytic cleavage of serum proteins to regulate successive enzymatic cascades or to generate specific protein fragments that can perform various functions. In particular, the plasminogen/plasmin system, which plays an important role in the dissolution of fibrin clots and remodelling of the extracellular matrix (ECM), utilizes a variety of proteolytic cleavage-based mechanisms to either positively or negatively regulate the activation of plasminogen to the serine protease plasmin (Cesarman-Maus and Hajjar, 2005). Briefly, plasmin is formed via the proteolytic processing of the plasma zymogen plasminogen by activators such as urokinase-type or tissue-type plasminogen activators (u-PA or t-PA) (Cesarman-Maus and Hajjar, 2005). Plasmin can in turn regulate plasminogen activation via a number of different mechanisms, namely (1) plasmin-mediated cleavage of single chain u-PA and t-PA to generate a more active two-chain enzyme (Nielsen et al., 1982; Pennica et al., 1983), (2) proteolytic cleavage of fibrin by plasmin to expose C-terminal lysine residues that enhance plasminogen binding to fibrin and activation by t-PA (Fleury et al., 1993),
and (3) plasmin-mediated cleavage of the u-PA receptor (u-PAR) to reduce the cell surface localization of u-PA for plasminogen activation (Montuori et al., 1999).

Histidine-rich glycoprotein (HRG), also known as histidine-proline-rich glycoprotein, is a ~75 kDa single polypeptide chain protein found in human plasma at the relatively high concentration of ~100-150 µg/ml (Jones et al., 2005a). HRG has a multi-domain structure consisting of two N-terminal regions with homology to cystatin-like domains (termed N1 and N2), a central histidine-rich region (HRR) flanked by two proline-rich regions (PRR1 and PRR2), and a C-terminal domain (Jones et al., 2005a). HRG also contains four intra-domain and two inter-domain disulfide bridges that link the C-terminal domain and the HRR to the N1N2 domain (Sorensen et al., 1993) (see Section 1.1.1 and Fig. 1.1). There are many ligands of HRG, such as Zn$^{2+}$, heparin, heparan sulfate (HS) and IgG, with HRG also interacting strongly with the lysine-binding sites on plasminogen (Lijnen et al., 1980), possibly via its C-terminal lysine residues (Saez et al., 1995; Borza and Morgan, 1997). Initially, HRG was proposed to be an anti-fibrinolytic agent by blocking plasminogen from interacting with binding partners that are important for plasmin activation (Lijnen et al., 1980), such as fibrinogen, fibrin, integrin $\alpha$M$\beta$2 and annexin 2 (Cesarman-Maus and Hajjar, 2005). However, there are conflicting reports in this area. Thus, some studies have demonstrated that HRG inhibits fibrinogen-dependent plasminogen activation (Borza et al., 2004), whereas others have shown no apparent effect of HRG on fibrin-dependent plasminogen activation in solution (Horne et al., 2000). In stark contrast, HRG has been suggested to function as a soluble plasminogen receptor that aids plasminogen activation by tethering plasminogen to glycosaminoglycan (GAG) coated surfaces (Borza et al., 2004) and cell surfaces (Jones et al., 2004a).

Human HRG has been shown to be susceptible to proteolytic cleavage in vitro by serine proteases such as plasmin and kallikrein but not thrombin (Smith et al., 1985). Extensive proteolysis of human HRG has also been observed in patients that have received streptokinase therapy, which increases plasmin activity in vivo (Smith et al., 1985). Furthermore, the ability of plasmin to cleave HRG at specific sites between different
domains of the molecule has been utilized to isolate various intact domains of HRG and characterize their structure and function, with rabbit HRG being studied in some detail (Borza et al., 1996; Borza and Morgan, 1997; Juarez et al., 2002; Donate et al., 2004). Although the release of the HRR of HRG following plasmin cleavage was suggested to play an important role in mediating the anti-angiogenic effects of the molecule (Olsson et al., 2004), the physiological significance of plasmin-mediated cleavage of human HRG has not been thoroughly examined and is the focus of this chapter.

4.3 Results

4.3.1 Proteolytic cleavage of human HRG$^{PID}$ by plasmin

Since the plasmin cleavage sites in human HRG have not been defined, the ability of plasmin to cleave human HRG was initially examined by SDS-PAGE under both non-reducing and reducing conditions. Under non-reducing conditions, plasmin-cleaved (30 to 120 mins at 37°C) HRG$^{PID}$ showed no major decrease in molecular weight compared to untreated (0 mins) HRG$^{PID}$, whereas multiple protein bands were observed under reducing conditions with plasmin-cleaved but not with untreated HRG$^{PID}$ (Fig. 4.1A). These results demonstrate that plasmin can effectively cleave human HRG at multiple sites and shows a similar cleavage pattern as previously described for rabbit HRG (Borza et al., 1996) and human HRG (Smith et al., 1985) when analyzed by SDS-PAGE under reducing conditions. Furthermore, plasmin-cleaved fragments of HRG remain predominately bound together by disulfide bonds and are not released from the rest of the molecule under non-reducing conditions. However, it is worth noting that under non-reducing conditions a small proportion of HRG$^{PID}$ that has been cleaved extensively by plasmin (120 mins at 37°C) showed a clear reduction in molecular weight compared to untreated (0 mins) HRG$^{PID}$ (Fig. 4.1A). These data suggest that certain plasmin-generated fragments of HRG are not linked to the rest of the molecule via disulfide bonds and they can be released from HRG under non-reducing conditions, although such fragments are only generated following prolonged plasmin-treatment.
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To determine the domains of HRG that are present in each fragment of plasmin-cleaved HRG, domain-specific rabbit anti-human HRG Abs (0115, 0116 and 0119) were used in Western blots to detect intact and plasmin-cleaved HRG under reducing conditions. As shown in Figure 4.1B, the N-terminal-specific anti-human HRG Ab (0116) reacted strongly with intact HRG$^{PID}$ and plasmin-cleaved HRG$^{PID}$ fragments that are ~37 kDa or above, indicating that all these fragments contain the N-terminal domain of HRG. It is worth noting that these N-terminal fragments of HRG$^{PID}$ appear to migrate as doublets on SDS-PAGE under reducing conditions (Fig. 4.1B), possibly representing different glycoforms of HRG. Moreover, the HRR-specific anti-human HRG Ab (0119) only detected intact HRG$^{PID}$ and the largest plasmin-cleaved HRG$^{PID}$ fragment of ~60 kDa, indicating that the epitope recognized by the HRR-specific anti-human HRG Ab (0119) is absent in all the other plasmin-cleaved HRG$^{PID}$ fragments (Fig. 4.1B). Similarly, the C-terminal-specific anti-human HRG Ab (0115) detected intact HRG$^{PID}$ and the ~12 kDa fragment (Fig. 4.1B), indicating that the ~12 kDa plasmin-cleaved HRG$^{PID}$ fragment contains the C-terminal domain of HRG.

The proteolytic cleavage of human HRG by plasmin was further investigated by Edman N-terminal sequence analysis of intact and selected plasmin-cleaved HRG fragments acquired under reducing conditions to define the plasmin cleavage sites and predict the domains of HRG present in the fragment of interest. The amino acid sequence of human HRG is shown in Figure 4.1C, with domains of HRG highlighted in different colours corresponding to Figure 4.1B and N-terminal sequencing results indicated in red. Consistent with the antibody binding results and previous studies on rabbit HRG, the N-terminal sequence of intact and the ~37 kDa fragment of HRG$^{PID}$ was identical (Fig. 4.1D), suggesting that all plasmin-cleaved HRG fragments above 37 kDa are likely to contain the same N-terminus and each successive fragment below 75 kDa represents progressive plasmin-mediated cleavage of HRG from the C-terminus. The ~12 kDa fragment that was released immediately after plasmin treatment of HRG$^{PID}$ at 37°C for 30 mins contains an N-terminal sequence of RRGPGKG (Fig. 4.1D), indicating a plasmin cleavage site on the carboxyl side of R421 and, therefore, this fragment contains
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Human HRG amino acid sequence

VSPTDCSAVEAEKALDLINKRRRDRGYLQQLRADDHALDRVEN  45
TTVYVLVDQGECVLSKRYWDDCEPPDSRRPSEVIGGKVI    90
ATRSHESQDLRVIDFTCNSSVSALNKTDSVLPVDVFFDTE   135
YRKOAKALEYKEENDDFASFRVDRRIERVARVREGGETG   178
DFSVRCHPRHHFPRHPNFVFGFRCRADFLYDEALDLESFKNLIV  222
CEVFDPGQEHINGVPHLGHPLFHVGWGHERSSTDKPBSFPGPHS 265
RDHHHPKHEHOOGHPOPDGERHONYHHPPQOPPLPMSCS     307
SCQHATFGTNGAQHRSHSNSSDLHPKHKHSHEQHPHGHHPH    349
AHPPHEHDTHRQPHGGHHPGHPPGHPGHPGHPHPHHPHPC     390
HDFQYGFCDDPPPQNHCHCQGQPPQHLRRRPGKGRPRP     431
FHCROQGYSVYRLPLRKGELPLPEANPSFPLPHKHPKPDKN 475
QFPQSVSSECGPKFSQGPQSVSMFTHTFFPK                 507

= NIN2   = PRR1/2   = HRR   = C   = Interdomain disulfide bridges

HRG# plasmin treatment (mins)
Western blot
Figure 4.1. Determination of plasmin cleavage sites in human HRG. (A) SDS-PAGE analysis of the proteolysis of HRG$^{PID}$ (10 μg, 1 mg/ml) by plasmin (0.5 μg, 50 μg/ml) at 37°C for 0, 30, 60 and 120 mins in the presence or absence of reducing agent DTT, with plasmin (0.5 μg) alone being included as a negative control. Protein bands were detected by 0.2% Coomassie Brilliant Blue (protein stain). (B) Western blot analysis of the proteolysis of HRG$^{PID}$ (750 ng, 1 mg/ml) by plasmin (37.5 ng, 50 μg/ml) at 37°C for 0, 30, 60 and 120 mins using the domain-specific rabbit anti-human HRG Abs (0115, 0116 and 0119), with the specificity of each Ab shown schematically in the upper panel. Histidine-rich sequences present in intact and plasmin-cleaved HRG$^{PID}$ (3 μg) were also detected by Western blot analysis using HRP-conjugated nickel nitrilotriacetic acid (Ni-NTA). (C) The amino acid sequence of human HRG, with N-terminal amino acid sequencing results acquired from intact and plasmin-cleaved HRG$^{PID}$ fragments indicated in red and domains of HRG highlighted in different colours. Cysteine residues that mediate interdomain disulfide bridges are indicated by (*). (D) N-terminal amino acid sequence analysis of intact and plasmin-cleaved HRG$^{PID}$ fragments obtained under reducing conditions. Edman N-terminal sequencing results for each HRG fragment are indicated in the Figure corresponding to the data acquired in (A). Based on the N-terminal sequencing results and Ab binding data acquired in (B), domains of HRG predicted to be present in each HRG fragment are also indicated in the Figure. (E) Schematic representation of the proteolysis of human HRG by plasmin, with red arrowheads indicating predicted plasmin cleavage sites. C, C-terminal domain; HRR, Histidine-rich region; N1, N-terminal domain 1; N2, N-terminal domain 2; PRR1, Proline-rich region 1; PPR2, Proline-rich region 2.
the C-terminal domain and parts of the PRR2 of HRG (Fig. 4.1C). Although no conclusive N-terminal sequence was obtained from the ~20 kDa plasmin-cleaved HRG$_{PID}$ fragment, histidine was found to be the most abundant amino acid in the first Edman sequencing cycle, followed by glycine, leucine and proline (Fig. 4.1D). These data suggest that the ~20 kDa fragment probably contains the HRR of HRG and has a frayed N-terminus. Furthermore, the presence of a frayed N-terminus in this ~20 kDa fragment could potentially disrupt the epitope recognized by the HRR-specific anti-human HRG Ab (0119) (Fig. 4.1B). These results are consistent with the difficulties encountered in isolating different domains of human HRG following plasmin digestion due to the presence of multiple internal plasmin cleavage sites within the HRR (Juarez et al., 2002). To validate whether the ~20 kDa fragment contains the HRR of HRG, HRP-conjugated nitrilotriacetic acid (Ni-NTA) was used to detect histidine-rich sequences present in various cleaved fragments of HRG. Surprisingly, similar to the HRR-specific anti-human HRG Ab (0119), Ni-NTA-HRP was found to bind predominately to intact HRG$_{PID}$ and the largest plasmin-cleaved HRG$_{PID}$ fragment of ~60 kDa, but not the ~20 kDa fragment (Fig. 4.1B). Thus, it is inconclusive from the current study whether the ~20 kDa fragment contains the HRR of HRG. Furthermore, it is worth noting that a faint band was also detected at ~30 kDa (Fig. 4.1A). However, due to the low yield of this fragment, its N-terminal sequence was not determined. Nevertheless, since the HRR- and C-terminal-specific anti-human HRG Abs (0119 and 0115) were able to recognize the ~30 kDa fragment (Fig. 4.1B), this fragment may contain both the HRR and C-terminal domain of HRG. Therefore, based on the data presented herein and previous studies on rabbit HRG (Borza et al., 1996; Juarez et al., 2002) and human HRG (Olsson et al., 2004), the predicted outcome of plasmin-mediated cleavage of HRG is shown in Figure 4.1E.

In order to monitor the effect of plasmin cleavage on various functions of HRG, an Ab-based detection method was developed. Since the N-terminal domains of HRG (i.e. the two cystatin-like domains, N1 and N2) are relatively resistant to proteases (Borza et al., 1996) and most plasmin-cleaved fragments of HRG are held together by the N1N2 domain under non-reducing conditions (see Fig. 4.1A above), a N1N2-specific anti-
human HRG mAb (HRG-4) was used to detect intact and plasmin-cleaved HRG in various assays. As detected by Western blot analysis under reducing conditions, anti-human HRG mAb (HRG-4) identified a stepwise reduction in the molecular weight of the N-terminus of HRG<sup>Pid</sup> following plasmin cleavage (Bands 1 to 4) (Fig. 4.2A). These results confirm that the generation of the lower molecular weight bands and the loss of the higher molecular weight bands represents the progressive plasmin-mediated cleavage of HRG from the C-terminus. The predicted identity of the N-terminal fragments of HRG (Bands 1 to 4) under reducing conditions are also shown in Figure 4.2A. Furthermore, the anti-human HRG mAb (HRG-4) effectively detected native intact (0 mins) and plasmin-cleaved (30 and 120 mins at 37°C) HRG<sup>Pid</sup> immobilized on ELISA wells to a similar extent (Fig. 4.2B). This implies that, under native conditions, the epitope on HRG recognized by the anti-human HRG mAb (HRG-4) is not sensitive to plasmin cleavage. Therefore, the Ab-based detection method provides a very sensitive means to detect intact and extensively plasmin-cleaved HRG.

### 4.3.2 Plasmin cleavage reduces HRG<sup>Pid</sup> binding to cell surface HS

The cleavage of HRG by plasmin has been shown to generate intact domains of HRG that have preserved functional properties (Borza <i>et al.</i>, 1996; Borza and Morgan, 1997). Although plasmin-cleaved HRG was predominantly used to isolate the different domains of HRG and to characterize the functions of each domain in isolation from the rest of the molecule (Borza <i>et al.</i>, 1996; Borza and Morgan, 1997; Juarez <i>et al.</i>, 2002; Donate <i>et al.</i>, 2004), plasmin cleavage can potentially play a physiological role in regulating the normal function of HRG by modulating HRG binding to various ligands. HRG binding to cell surface HS has been shown to play an important role in regulating growth factor binding to HS (Brown and Parish, 1994) as well as tethering plasminogen to the cell surface (Borza <i>et al.</i>, 2004; Jones <i>et al.</i>, 2004a). Thus, the effect of plasmin cleavage on HRG binding to cell surface HS was initially examined, with viable CHO-K1 cells being used as the model cell line for cell surface HS binding (Jones <i>et al.</i>, 2004b). As shown in Figure 4.3A, HRG<sup>Pid</sup> (100 μg/ml) bound strongly to viable CHO-K1 cells and showed...
Figure 4.2. Analysis of plasmin-cleaved HRG<sup>PID</sup> using a monoclonal antibody specific for the N1N2 domain. (A) Western blot analysis of the proteolysis of HRG<sup>PID</sup> (300 ng, 300 μg/ml) by plasmin (30 ng, 30 μg/ml) at 37°C for 0 to 120 mins using the N1N2-specific mouse anti-human HRG mAb (HRG-4), with the predicted identity of each N-terminal fragment of HRG under reducing conditions shown on the right (Bands 1 to 4). (B) Analysis of the ability of the mouse anti-human HRG mAb, HRG-4 (0.6 ng/ml), to bind to ELISA wells pre-coated with different concentrations (0.08 to 5 μg/ml) of intact HRG<sup>PID</sup> (0 mins) or HRG<sup>PID</sup> plasmin-cleaved at 37°C for 30 and 120 mins, with plasmin alone being included as a negative control. Error bars represent SEM (n = 3).
Figure 4.3. Plasmin cleavage reduces the ability of HRG\textsuperscript{PID} to bind to cell surface HS. (A) Ability of HRG\textsuperscript{PID} (100 µg/ml) to bind viable CHO-K1 cells and a GAG deficient CHO cell line (pgsA-745) as detected by flow cytometry using a mouse anti-human HRG mAb (HRG-4) and a FITC-conjugated sheep F(ab')\textsubscript{2} anti-mouse Ig Ab. Representative flow cytometry histograms are shown, with filled histograms representing primary and secondary Ab only control, and open black histograms representing HRG binding. (B) Effect of plasmin cleavage (0, 30 and 120 mins at 37°C) on HRG\textsuperscript{PID} (100 µg/ml) binding to viable CHO-K1 cells at pH 7.2 in the presence or absence of heparin (12.5 kDa, 100 µg/ml). (C) Effect of Zn\textsuperscript{2+} (20 µM) and acid pH (pH 6.6) on the binding of 100 µg/ml of intact (0 mins) or plasmin-cleaved (30 and 120 mins at 37°C) HRG\textsuperscript{PID} to viable CHO-K1 cells. HRG binding in (B) and (C) was analyzed by flow cytometry as described in Figure 3.4, with data being expressed as fold binding above background MFI. Error bars represent SEM (n = 3). NS, not significant. **, \(P < 0.01\). ***, \(P < 0.001\).
no detectable binding to the GAG deficient CHO cell line pgsA-745, indicating that HRG binding to CHO-K1 cells is mediated via cell surface GAG, such as HS (Jones et al., 2004b). HRG$^{PID}$ treated with plasmin at 37°C for 0, 30 and 120 mins was used to represent intact HRG, HRG that has been cleaved predominately at position R421 or extensively cleaved HRG, respectively. Strikingly, HRG$^{PID}$ (100 μg/ml) binding to cell surface HS was reduced by >50% when HRG$^{PID}$ was treated with plasmin for either 30 or 120 mins at 37°C (Fig. 4.3B). These results suggest that plasmin cleavage can potentially disrupt the HS binding site and/or modify the conformation of HRG to reduce HRG binding to cell surface HS. It is worth noting that initial (30 mins at 37°C) or extensive (120 mins at 37°C) plasmin cleavage of HRG$^{PID}$ had a similar level of inhibitory effect (~55%) on HRG$^{PID}$ binding to CHO-K1 cells (Fig. 4.3B), indicating that a single plasmin cleavage at position R421 is adequate to significantly reduce HRG binding to cell surface HS and further plasmin cleavage had no additional effect. Furthermore, the presence of heparin (12.5 kDa, 100 μg/ml) completely abolished the ability of intact (0 mins) as well as plasmin-cleaved (30 and 120 mins at 37°C) HRG$^{PID}$ to bind to CHO-K1 cells (Fig. 4.3B), indicating that both intact and plasmin-cleaved HRG can still bind to heparin, which competes for cell surface HS binding.

Previous studies have demonstrated that the binding of HRG to cell surfaces can be potentiated by the presence of Zn$^{2+}$ (Olsen et al., 1996; Jones et al., 2004b) or at low pH (Jones et al., 2004a). Therefore, the ability of plasmin-cleaved HRG to bind to CHO-K1 cells in the presence of Zn$^{2+}$ or at acidic pH was also investigated. Consistent with previous studies, the presence of physiological concentrations of Zn$^{2+}$ (20 μM) or pH 6.6 enhanced the binding of HRG$^{PID}$ (100 μg/ml) to cell surface HS (Fig. 4.3C). Interestingly, plasmin treatment (30 and 120 mins at 37°C) not only reduced HRG$^{PID}$ binding to CHO-K1 cells, but also abolished the ability of Zn$^{2+}$ or acidic pH to enhance HRG$^{PID}$ binding (Fig. 4.3C), suggesting that an intact molecule is required for HRG to respond to regulatory factors such as Zn$^{2+}$ and pH. Thus, plasmin cleavage can potentially inhibit the ability of HRG to function as a pH and Zn$^{2+}$ sensor in response to tissue injury.
Chapter 4

In additional experiments, the ability of intact and plasmin-cleaved HRG to bind to necrotic Jurkat T cells was also examined. In contrast to cell surface HS binding, plasmin-mediated cleavage significantly enhanced the binding of HRG\textsuperscript{PID} (100 µg/ml) to necrotic cells (Fig. 4.4). These data suggest that plasmin cleavage at sites of tissue injury may potentiate HRG binding to necrotic cells. Moreover, consistent with Section 3.3.10 and Jones et al. (2005b), the interaction between HRG and necrotic cells is likely to be independent of cell surface HS since, despite plasmin cleavage affecting the binding of HRG to cell surface HS on viable cells, plasmin cleavage had no inhibitory effect on HRG binding to necrotic cell.

**Figure 4.4.** Plasmin cleavage potentiates the bind of HRG\textsuperscript{PID} to necrotic Jurkat T cells. Heat induced necrotic Jurkat T cells (56°C, 30 mins) were incubated with 100 µg/ml of intact (0 mins) HRG\textsuperscript{PID} or plasmin-cleaved (30 and 120 mins at 37°C) HRG\textsuperscript{PID}. HRG binding was detected by flow cytometry as described in Figure 3.4, with data being expressed as fold binding above background MFI. Error bars represent SEM (n = 3). **, P < 0.01.

4.3.3 Plasmin cleavage may modulate the ability of HRG\textsuperscript{PID} to regulate the plasminogen/plasmin system

HRG has been shown by a number of studies to bind to plasminogen (Lijnen et al., 1980; Saez et al., 1995; Borza and Morgan, 1997; Borza et al., 2004; Jones et al., 2004a)
and modulate the activation of plasminogen to plasmin (Borza and Morgan, 1997; Borza et al., 2004). The ability of HRG to tether plasminogen to GAG coated surfaces has also been proposed to play an important role in facilitating plasminogen activation (Borza et al., 2004; Jones et al., 2004a). Since HRG itself is sensitive to plasmin cleavage, activation of plasminogen to plasmin may in turn promote the generation of plasmin-cleaved HRG and alter the ability of HRG to regulate the plasminogen/plasmin system. Therefore, the effect of plasmin cleavage on the binding of HRG to plasminogen was initially investigated. Unlike cell surface HS binding, initial plasmin cleavage (30 mins at 37°C) markedly enhanced HRG\textsuperscript{PID} binding to plasminogen immobilized on ELISA wells, whereas extensive plasmin cleavage (120 mins at 37°C) of HRG\textsuperscript{PID} resulted in plasminogen binding returning to control levels (Fig. 4.5A). These data suggest that a single plasmin cleavage at position R421 may alter the conformation of the molecule and expose C-terminal lysine residues to aid plasminogen binding. In contrast, extensive plasmin cleavage may further modify the conformation of HRG and/or remove C-terminal lysine residues to abolish the enhanced plasminogen binding induced by initial plasmin cleavage.

Furthermore, to examine the effect of plasmin cleavage on the ability of HRG to tether plasminogen to GAG coated surfaces, intact (0 mins) and plasmin-cleaved (30 and 120 mins at 37°C) HRG\textsuperscript{PID} were either pre-coated on heparin coated plates prior to assessing plasminogen (5 μg/ml) binding (Fig. 4.5B) or were incubated simultaneously with plasminogen on heparin coated wells (Fig. 4.5C). As expected, plasminogen binding to heparin coated wells was enhanced markedly by intact (0 mins) HRG\textsuperscript{PID} using either binding procedure (Fig. 4.5B and C). Surprisingly, plasmin cleavage had minimal effect on the ability of HRG\textsuperscript{PID} to tether plasminogen to heparin coated wells (Fig. 4.5B and C), indicating that both intact and plasmin-cleaved HRG can enhance the binding of plasminogen to GAG coated surfaces.
Figure 4.5. Effect of plasmin cleavage on the ability of HRG^{PID} to bind to plasminogen and tether plasminogen to immobilized heparin. (A) Effect of plasmin cleavage (0, 30 and 120 mins at 37°C) on HRG^{PID} binding to immobilized human plasminogen by ELISA using wells pre-coated with 1 μg/ml of human plasminogen. Error bars represent SEM (n = 3). (B) Analysis of the ability of intact (0 mins) and plasmin-cleaved (30 and 120 mins at 37°C) HRG^{PID} to tether human plasminogen to heparin coated ELISA wells by pre-coating heparin coated wells with HRG^{PID} prior to assessing human plasminogen (5 μg/ml) binding or (C) by simultaneous incubation of HRG^{PID} and human plasminogen (5 μg/ml) with heparin coated wells. ELISA wells were pre-coated with 10 μg/ml of ExtrAvidin® and 10 μg/ml of biotinylated heparin, and then analyzed for plasminogen binding by ELISA under the different conditions. Error bars represent the range of duplicate samples.
4.3.4 Regulation of plasmin-mediated cleavage of HRG<sub>PID</sub> by heparin, Zn<sup>2+</sup> and pH

Since plasmin cleavage can regulate various functions of HRG (see above), factors that may modulate the proteolysis of HRG by plasmin were further examined. Firstly, the binding of growth factors and chemokines such as fibroblast growth factor (FGF) (Saksela et al., 1988) and eotaxin (Ellyard et al., 2007) to heparin/HS has been shown to protect these molecules from plasmin-mediated degradation. Thus, the effect of heparin on the proteolytic cleavage of HRG by plasmin was investigated to evaluate the sensitivity of HRG to plasmin cleavage when bound to cell surface HS or free heparin. As shown in Figure 4.6A, heparin (12.5 kDa, 2 and 10 μg/ml) alone did not interfere with plasmin activity as measured by the cleavage of the chromogenic substrate N-(p-Tosyl)-Gly-Pro-Lys. However, the presence of heparin (12.5 kDa, 5 μg/ml) attenuated plasmin-mediated cleavage of HRG<sub>PID</sub>, as indicated by the lack of lower molecular weight N-terminal fragments of HRG<sub>PID</sub> (Fig. 4.6B and C). These data suggest that the binding of HRG to heparin can protect HRG from plasmin cleavage, possibly by masking exposed plasmin cleavage sites.

Furthermore, an increase in the local concentration of Zn<sup>2+</sup> and a decrease in pH at sites of tissue injury have been proposed to regulate various functions of HRG, such as HRG tethering plasminogen to cell surfaces (Jones et al., 2004a) and HRG exerting antibacterial effects (Rydengard et al., 2007; Rydengard et al., 2008). Thus, the effect of Zn<sup>2+</sup> and acidic pH on the proteolysis of HRG by plasmin was investigated. Acidic pH (in particular pH 6.0), and to a much lesser extent the presence of Zn<sup>2+</sup> (10 and 20 μM), reduced the enzymatic activity of plasmin against a chromogenic substrate (Fig. 4.7A), but also resulted in a substantial reduction in plasmin-mediated cleavage of HRG<sub>PID</sub> (Fig. 4.7B to E). These results suggest that an increase in the local Zn<sup>2+</sup> concentration and/or a decrease in pH may delay plasmin-mediated cleavage of HRG by directly reducing plasmin activity but, potentially in the case of Zn<sup>2+</sup>, by rendering HRG less susceptible to plasmin cleavage, possibly via a conformational change in the HRG molecule.
Figure 4.6. Heparin protects HRG\textsuperscript{PID} from plasmin cleavage. (A) Effect of heparin (12.5 kDa, 2 or 10 \mu g/ml) on plasmin enzymatic activity (2 \mu g/ml) as measured by the cleavage of the chromogenic substrate N-(p-Tosyl)-Gly-Pro-Lys (1 mg/ml) at 37°C for 60 mins. Error bars represent SEM (n = 3). (B) Western blot analysis of the proteolysis of HRG\textsuperscript{PID} (300 ng, 30 \mu g/ml) by plasmin (60 ng, 6 \mu g/ml) at 37°C for 60 mins in the presence or absence of heparin (12.5 kDa, 50 ng, 5 \mu g/ml). N-terminal fragments of HRG were analyzed by SDS-PAGE under reducing conditions and were detected by Western blot using the mouse anti-human HRG mAb (HRG-4). (C) Effect of heparin (12.5 kDa) on plasmin-mediated proteolysis of HRG\textsuperscript{PID} was quantified by densitometry analysis of the N-terminal fragments of HRG based on the data from (B). Predicted N-terminal fragments of HRG (Bands 1 to 4) as in Figure 4.2A.
Figure 4.7. Effect of pH and Zn^{2+} on proteolytic cleavage of HRG^{PID} by plasmin. (A) Effect of pH and Zn^{2+} on plasmin enzymatic activity (2 μg/ml) as measured by the cleavage of the chromogenic substrate N-(p-Tosyl)-Gly-Pro-Lys (1 mg/ml) at 37°C for 60 mins. Error bars represent SEM (n = 3). (B) Western blot analysis of the proteolysis of HRG^{PID} (300 ng, 30 μg/ml) by plasmin (60 ng, 6 μg/ml) at 37°C for 60 mins in the presence of Zn^{2+} (10 or 20 μM) and at different pH (pH 6.0, 6.6 or 7.2). N-terminal fragments of HRG were analyzed by SDS-PAGE under reducing conditions and were detected using the mouse anti-human HRG mAb (HRG-4). Effect of Zn^{2+} on the proteolysis of HRG^{PID} by plasmin at (C) pH 6.0, (D) pH 6.6 and (E) pH 7.2 was quantified by densitometry analysis of the N-terminal fragments of HRG based on the data from (B). Predicted N-terminal fragments of HRG (Bands 1 to 4) as in Figure 4.2A.
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A

Plasmin activity

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Zn^{2+}

B

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HRGPID

Band 1

Band 2

Band 3

Band 4

Zn^{2+} (μM)

pH 6.0

pH 6.6

pH 7.2

C

Plasmin treatment at pH 6.0

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Zn^{2+}

D

Plasmin treatment at pH 6.6

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Zn^{2+}

E

Plasmin treatment at pH 7.2

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Zn^{2+}
4.4 Discussion

Based on the modular structure of HRG and the ability of HRG to bind to a variety of different ligands (Jones et al., 2005a), it has been proposed that HRG may function as an adaptor molecule that interacts with multiple ligands simultaneously via several independent binding sites (Borza et al., 1996). Thus, HRG can potentially regulate numerous biological processes such as cell adhesion, angiogenesis, coagulation, fibrinolysis and immune complex/necrotic cell/pathogen clearance (see Section 1.1). Importantly, the function of HRG is often regulated by pH and Zn$^{2+}$ (Jones et al., 2005a), these factors modulating HRG activity at sites of tissue injury when local pH decreases (Punnia-Moorthy, 1987; LaManna, 1996) or when local Zn$^{2+}$ concentrations increase due to the release of Zn$^{2+}$ from degranulating platelets (Aktulga, 1974). Apart from local Zn$^{2+}$ concentrations and pH, it has been suggested that proteolytic degradation of HRG may be required for HRG to exert its anti-angiogenic activity (Olsson et al., 2004). Although the ability of proteases such as plasmin and kallikrein to cleave HRG was initially reported by Smith et al. (1985), the results presented in this chapter provide the first detailed analysis of the proteolytic cleavage of human HRG by plasmin and demonstrate the potential effects of plasmin-mediated cleavage on the functional activity of HRG (summarized in Fig. 4.8).

In contrast to kallikrein-mediated cleavage of HRG, which degrades HRG extensively and rapidly (Smith et al., 1985), HRG is cleaved at specific sites by plasmin generating distinct fragments that are relatively resistant to further plasmin cleavage, with the exception that an internal ~20 kDa fragment is degraded following prolonged plasmin treatment (Fig. 4.1). Most importantly, plasmin cleavage occurs between the various domains of HRG, which may preserve the function of these domains (e.g. the N1N2, HRR and C-terminal domain) (Fig. 4.1). However, unlike the closely related cystatin superfamily member high-molecular-weight kininogen (HMWK) (Kleniewski and Donaldson, 1987; Kleniewski et al., 1992), the majority of the plasmin-generated
Figure 4.8. Regulation of HRG function by plasmin-mediated proteolytic cleavage.

**HRG prior to plasmin cleavage**
- HRG binds strongly to cell surface HS
- HRG binding to cell surface HS is potentiated by acidic pH and Zn$^{2+}$
- Heparin binding, acidic pH and elevated Zn$^{2+}$ inhibits plasmin-mediated cleavage of HRG

**Initial plasmin cleavage of HRG**
- HRG binding to cell surface HS is reduced and becomes less responsive to acidic pH and Zn$^{2+}$
- HRG binding to plasminogen is enhanced and may act as a decoy receptor for soluble plasminogen
- Increased HRG binding to necrotic cells

**Extensive plasmin cleavage of HRG**
- Generation of HRG fragments that may have various biological activities (e.g. anti-angiogenic and anti-microbial properties)
fragments of HRG remain bound to the N1N2 domain of HRG via disulfide bridges (Fig. 4.1), suggesting that plasmin cleavage alone is unlikely to release various potentially active fragments of HRG.

It is worth noting that since intact HRG was used in most of the published studies that have defined a number of HRG functions, plasmin-mediated cleavage is unlikely to be essential for HRG to perform its role in these biological processes. However, the potential cleavage of interdomain disulfide bonds of HRG (i.e. Cys6-Cys486 and Cys185-Cys399) in the extracellular environment via dithiol-disulfide exchange, alkaline hydrolysis or acid-based assisted hydrolysis (Hogg, 2003), may play a critical role in modulating the functional activity of plasmin-cleaved HRG in multiple biological systems. For example, under normal physiological conditions, a large proportion of HRG may form high affinity complexes with ligands such as IgG, plasminogen and HS present on cell surfaces and in the ECM. These complexes can potentially sequester HRG from interacting with other ligands or inducing an anti-angiogenic, anti-microbial or endotoxin neutralizing effect via the HRR of HRG. Therefore, the combination of proteolytic and disulfide cleavage may release fragments of HRG containing different domains that can perform independent functions. Alternatively, the release of different cleaved fragments of HRG may also abolish the ability of HRG to function as an adaptor molecule, such as tethering plasminogen to GAG surfaces (Borza et al., 2004; Jones et al., 2004a). Interestingly, similar to the disulfide bond in domain 2 of CD4 (Matthias et al., 2002), the interdomain disulfide bond between the HRR and N2 domain of HRG (Cys185-Cys399) was predicted to be susceptible to reductive cleavage due to the unusual geometry and strain of this disulfide bond (P. Hogg, unpublished observations). Thus, it seems likely that the function of HRG is regulated by both disulfide and proteolytic cleavage. The co-operative effect of proteolytic and disulfide cleavage has also been reported for the generation of angiostatin and microplasmin from plasmin, which involves the reduction of disulfide bonds in the kringle 5 domain of plasmin prior to proteolytic cleavage (Statakis et al., 1997; Statakis et al., 1999).
In addition to the requirement for disulfide cleavage to release various active fragments from plasmin-cleaved HRG, the data presented in this chapter suggest that plasmin cleavage alone can also modulate the ability of HRG to bind to various ligands, possibly by altering the conformation of HRG and/or disrupting ligand binding sites. In this study, a single plasmin cleavage at R421 on HRG was sufficient to reduce HRG binding to cell surface HS by >50% (Fig. 4.3B). Since the R421 plasmin cleavage site is located distantly from the two proposed HS/heparin binding sites on HRG (i.e. the NIN2 domain (Jones et al., 2004b) and HRR (Vanwildemeersch et al., 2006) of HRG), the decrease in HRG binding to cell surface HS following plasmin cleavage at a single site is likely to be the result of a conformational change in HRG, rather than being due to a direct disruption of the HS/heparin binding sites on HRG. It is worth noting that plasmin cleavage only partially reduced the binding of HRG to HS (Fig. 4.3B), suggesting that one or both HS/heparin binding sites on HRG can remain (partly) functional following plasmin cleavage. However, some indirect evidence presented in this chapter suggests that plasmin cleavage is likely to modify the function of the HS/heparin binding site located in the HRR rather than the NIN2 domain of HRG. For example, excessive plasmin cleavage (120 mins at 37°C), which preserved the integrity of the NIN2 domain but degraded the predicted HRR fragment of HRG (Fig. 4.1), resulted in no further reduction in HRG binding to cell surface HS compared to HRG that was cleaved predominately at R421 (Fig. 4.3B and C). Furthermore, plasmin cleavage also rendered HRG non-responsive to acidic pH or Zn\(^{2+}\) (Fig. 4.3C), which are factors known to potentiate the HS/heparin binding properties of the HRR (Vanwildemeersch et al., 2006) but not the NIN2 domain of HRG (Jones et al., 2004b). Thus, the residual binding of HRG to cell surface HS and heparin following plasmin cleavage is likely to be mediated via the NIN2 domain of HRG.

Since HS binding is required for HRG to regulate various biological processes (see Section 1.1), the ability of plasmin cleavage to modulate HRG binding to HS is likely to have a major impact on the function of HRG. For example, plasmin cleavage may affect the ability of HRG to compete with other HS/heparin-binding proteins, such as FGF or heparanase, interacting with cell surface HS and the ECM. Previous studies have also
demonstrated that HRG can aid the activation of plasminogen to plasmin by tethering plasminogen to GAG surfaces (Borza et al., 2004; Jones et al., 2004a). Thus, proteolytic cleavage of HRG by plasmin may act as a negative feedback mechanism to limit further plasminogen activation by reducing the amount of intact HRG that can efficiently tether plasminogen to GAG surfaces. Interestingly, similar to plasmin-cleaved fibrin (Fleury et al., 1993), initially plasmin cleavage enhanced the binding of HRG to plasminogen (Fig. 4.5A), possibly via the exposure of C-terminal lysine. This is consistent with previous studies where ‘plasmin-clipped’ rabbit HRG also shows an enhancement in plasminogen binding compared to intact HRG (Borza and Morgan, 1997). Since plasmin-cleaved HRG retained the ability to bind plasminogen (Fig. 4.5A), but interacted with cell surface HS less efficiently (Fig. 4.3), plasmin-cleaved HRG may also act as a decoy receptor for plasminogen, which could compete with intact HRG or other plasminogen receptors for plasminogen binding to cell surfaces. Surprisingly, plasmin cleavage did not have any apparent effect on the ability of HRG to tether plasminogen to heparin coated surfaces (Fig. 4.5B and C), although this may be due to the high density of immobilized heparin masking the partial loss in cell surface HS binding exhibited by plasmin-cleaved HRG.

As mentioned above (see also Section 1.1), several studies have suggested that the HRR of HRG, in particular peptides derived from the HRR of HRG, exhibit anti-angiogenic (Juarez et al., 2002; Donate et al., 2004; Olsson et al., 2004; Dixelius et al., 2006; Lee et al., 2006), anti-microbial (Kacprzyk et al., 2007; Rydengard et al., 2007; Rydengard et al., 2008) as well as endotoxin neutralizing properties (Bosshart and Heinzelmann, 2003). Interestingly, unlike rabbit HRG, a potential plasmin cleavage site at R360 within the HRR of human HRG may allow the release of a HRG fragment in the absence of disulfide cleavage (corresponding to amino acid residues 322-360 following plasmin cleavage at R321 and R360) that is similar to the well characterized HRG derived heparin-binding/anti-angiogenic peptide, HRGP330 (HRG residues 330-364) (Dixelius et al., 2006; Lee et al., 2006; Vanwildemeersch et al., 2006). Although this predicted HRR fragment of HRG was not apparent in this study when plasmin-cleaved HRG was analyzed under both non-reducing and reducing conditions (Fig. 4.1), further
investigation is needed to determine whether the release of the HRR of HRG requires both proteolytic and disulfide cleavage. Interestingly, unpublished observations suggest that elastase is capable of digesting HRG and may generate peptides derived from the HRR of HRG (Rydengard et al., 2007).

The experimental data presented in this chapter also demonstrate that factors such as heparin (Fig. 4.6), Zn$^{2+}$ and pH (Fig. 4.7), may co-operatively regulate the ability of plasmin to cleave HRG. Consistent with an earlier study by Smith et al. (1985), the binding of heparin by HRG reduced the sensitivity of HRG to plasmin cleavage (Fig. 4.6). Similarly, acidic pH and the presence of Zn$^{2+}$ directly inhibited plasmin activity, as well as substantially reducing the cleavage of HRG by plasmin (Fig. 4.7). Indeed, the ability of Zn$^{2+}$ to inhibit plasmin activity has been shown previously to abolish the proteolytic cleavage of fibrinogen by plasmin (Nowak and Zgirski, 2003). In addition, under conditions of tissue injury when the local concentration of Zn$^{2+}$ is elevated (Aktulga, 1974) and the pH is acidic (Punnia-Moorthy, 1987; LaManna, 1996), HRG can bind more effectively to cell surface HS (Jones et al., 2004a; Jones et al., 2004b) thereby masking plasmin cleavage sites and possibly further delaying the proteolytic cleavage of HRG by plasmin. Based on these data, the ability of acidic pH and elevated Zn$^{2+}$ levels to reduce plasmin-mediated cleavage of HRG represents a novel indirect mechanism of regulating the function of HRG.

In summary, the experimental data presented in this chapter demonstrate that various functions of HRG can be regulated by plasmin cleavage, which may in turn affect the ability of HRG to modulate the plasminogen/plasmin system. Since proteolytically cleaved HRG can persist in the circulation (Smith et al., 1985), cleavage of HRG by proteases such as plasmin may provide an elegant means of regulating the multifunctional properties of HRG, rather than simply controlling the turnover of the protein. Further investigations are needed to examine the role of plasmin cleavage, as well as proteolytic cleavage by other proteases such as kallikrein and elastase, in regulating the function of HRG in immunity, tumour progression and vascular biology.
5.1 Abstract

Heparanase, an endo-β-D-glucuronidase, is involved in numerous normal physiological and pathological processes through its ability to mediate the degradation of heparan sulfate (HS), a key structural component of the extracellular matrix (ECM) and on the surface of cells. Human heparanase is synthesized as an inactive 65 kDa latent precursor, which undergoes proteolytic cleavage to yield 8 and 50 kDa subunits that heterodimerize into an active enzyme. The ability of the extracellular heparanase precursor to tether onto cell surface heparan sulfate proteoglycans (HSPG) and other receptor(s), such as the cation-independent mannose-6-phosphate receptor (CIMPR), is key to its activation, function and uptake into intracellular compartments. This chapter describes experiments demonstrating that a relatively abundant plasma glycoprotein, histidine-rich glycoprotein (HRG), interacts directly with heparanase and interferes with heparanase binding to cell surface receptors, particularly HSPG. The presence of HRG also enhances heparanase
enzymatic activity. Thus, the direct interaction between HRG and heparanase can potentially regulate the role of heparanase in health and disease.

5.2 Introduction

Structures of the extracellular environment are fundamental in providing a molecular scaffolding for cells within different tissues as well as regulating numerous biological processes such as cell proliferation, differentiation, adhesion and migration (Bernfield et al., 1999). Besides proteins such as laminin and collagen, heparan sulfate proteoglycans (HSPG), which are composed of a protein core and linear side-chains of the complex glycosaminoglycan heparan sulfate (HS), are ubiquitous macromolecules that can be found within the extracellular milieu of a variety of tissues and play an important role in aiding the assembly of the extracellular matrix (ECM) (Kjellen and Lindahl, 1991). HSPG can also be found on cell surfaces and participate in binding a variety of bioactive molecules (e.g. growth factors, cytokines, chemokines, coagulation factors) via the negatively charged HS side-chains, which subsequently regulate the availability and function of these molecules (Bernfield et al., 1999). In conjunction with various proteases, heparanase, an endo-β-D-glucuronidase, has been proposed to participate in the remodelling and degradation of the ECM via HS cleavage (Bar-Ner et al., 1986). Furthermore, cleavage of the HS side-chains of HSPG by heparanase can also liberate HS-bound molecules and make them functionally available (Ishai-Michaeli et al., 1990; Elkin et al., 2001; Myler and West, 2002; Reiland et al., 2006). Based on these functional properties, heparanase has been implicated in numerous normal physiological and pathological processes such as angiogenesis, wound healing, cell migration as well as tumour metastasis (Parish et al., 2001).

Human heparanase is synthesized as an inactive 65 kDa latent precursor, which undergoes proteolytic cleavage to release a 6 kDa linker fragment and become an active enzyme consisting of a heterodimer between the remaining 8 and 50 kDa subunits (Levy-Adam et al., 2003). The ability of extracellular heparanase to bind efficiently to cell surfaces has been suggested to regulate various key aspects of heparanase functions.
Chapter 5

(Parish et al., 1998; Parish et al., 2001; Ilan et al., 2006; Vreys and David, 2007), such as cellular uptake, processing and storage of heparanase (Nadav et al., 2002; Gingis-Velitski et al., 2004b; Zetser et al., 2004; Vreys et al., 2005; Ben-Zaken et al., 2007; Wood and Hulett, 2008), triggering signalling cascades (Gingis-Velitski et al., 2004a; Sotnikov et al., 2004; Zetser et al., 2006), facilitating cell adhesion to the ECM (Gilat et al., 1995; Goldshmidt et al., 2003; Sotnikov et al., 2004), as well as degradation of the ECM to aid cell migration (Sasaki et al., 2004; Benhamron et al., 2006; Wood and Hulett, 2008). To date, HSPG, low density lipoprotein receptor-related protein (LRP) and the cation independent mannose-6-phosphate receptor (CIMPR) have been identified as cell surface receptors for heparanase (Nadav et al., 2002; Gingis-Velitski et al., 2004b; Vreys et al., 2005; Ben-Zaken et al., 2008; Wood and Hulett, 2008). In addition to cell surface receptors regulating the function of heparanase, soluble eosinophil major basic protein (MBP) has also been proposed as a natural heparanase binding protein that can block heparanase activity (Temkin et al., 2004). Studies by Temkin et al. (2004) reported that MBP, an abundant cationic protein localized in the core of eosinophil secondary granules, partially co-localizes with heparanase in human eosinophils, co-immuno-precipitates with heparanase from eosinophil lysates and inhibits heparanase activity. Unexpectedly, histidine-rich glycoprotein (HRG), an abundant multi-functional plasma protein (Jones et al., 2005a), was found to co-purify with human platelet-derived heparanase. Therefore, this chapter describes experiments which further examined the direct interaction between HRG and heparanase, as well as the effect of HRG on heparanase binding to cell surface receptors and on heparanase enzymatic activity.

5.3 Results

5.3.1 HRG co-purifies with human platelet-derived heparanase

As described earlier in Section 1.1.3.2 and 4.2, HRG binds plasminogen with high affinity (Lijnen et al., 1980; Saez et al., 1995; Borza and Morgan, 1997; Borza et al., 2004; Jones et al., 2004a) and aids the activation of plasminogen to generate the serine
protease plasmin (Borza and Morgan, 1997; Borza et al., 2004). In addition to fibrinolysis, the plasminogen/plasmin system has also been implicated in degradation of the ECM to assist various normal physiological and pathological processes (Castellino and Ploplis, 2005). Thus, HRG may play an important role in regulating the remodelling of the ECM during development, angiogenesis, cell migration as well as cancer metastasis (see Section 1.1). Besides plasminogen, unpublished data in our laboratory has also observed that HRG may interact either directly or indirectly with another matrix degrading enzyme, the endo-β-D-glucuronidase heparanase (Freeman C., personal communication). Prior to investigating whether any direct interaction between HRG and heparanase occurs, the purity of HRG\textsuperscript{PID} and human platelet-derived heparanase was first examined. Western blot analysis of the heparanase content in HRG\textsuperscript{PID} (10 μg) was performed and showed no apparent co-purification of heparanase (Fig. 5.1A). However, the anti-human HRG mAb (HRG-4) was found to bind specifically to human platelet-derived heparanase (1 μg/ml) immobilized on ELISA plates compared to a BSA only control (Fig. 5.1B). Since HRG has been found in the α-granules of platelets and megakaryocytes (Leung 1983), these results suggested that HRG can potentially co-purify with human platelet-derived heparanase. Using two different anti-human HRG mAb (HRG-3 and HRG-4), fragments of HRG (~45-50 kDa) were found to be present in the heparanase preparation (1 μg) (Fig. 5.1C). It is worth noting that HRG is not always detectable in every heparanase preparation examined (data not shown), suggesting that the purification process can occasionally disassociate HRG from the heparanase. Nevertheless, HRG can be depleted from heparanase preparations by passage of the preparations through a 100 kDa cut off Centricon filter and such HRG-free heparanase preparations were used for the rest of this study.

### 5.3.2 HRG\textsuperscript{PID} directly interacts with heparanase

Based on the above observations, the ability of HRG to interact directly with heparanase was examined via ELISA. Intact HRG\textsuperscript{PID} was able to bind to heparanase immobilized on ELISA plates in a concentration dependent and saturable manner (Fig. 5.2A), with
Figure 5.1. HRG co-purifies with human platelet-derived heparanase. (A) Western blot analysis of the heparanase content of $HRG^{PID}$ (10 $\mu$g), with heparanase (200 $\mu$g) being included as a positive control. (B) Analysis of the ability of a mouse anti-human HRG mAb (HRG-4) to bind to ELISA wells pre-coated with nothing or 1 $\mu$g/ml of human platelet-derived heparanase prior to blocking with 3% BSA. (C) Western blot analysis of the HRG content of heparanase (1 $\mu$g) using two different mouse anti-human HRG mAb, HRG-3 or HRG-4, with a proteolytic-cleaved $HRG^{PID}$ preparation (250 $\mu$g) being included as a positive control. Mouse anti-rat CD90 mAb (Thy 1.1) is included as a mouse IgG1 isotype control.
Figure 5.2. HRG$^{PID}$ binds heparanase, an interaction that is not affected by plasmin-mediated proteolytic cleavage of HRG$^{PID}$. (A) Analysis of the ability of HRG$^{PID}$ to bind to ELISA wells pre-coated with 1 µg/ml of heparanase at pH 7.2. Error bars represent SEM (n = 3). (B) Ability of HRG$^{PID}$ to bind to ELISA wells pre-coated with 2 µg/ml of heparanase, OVA, ExtraAvidin® or BSA only control at pH 7.2. (D) Effect of plasmin cleavage (0, 30, 60 and 120 mins at 37°C) on HRG$^{PID}$ binding to ELISA wells pre-coated with 2 µg/ml of heparanase at pH 7.2. Error bars represent the range of duplicate samples.
binding being heparanase-specific compared with the control proteins OVA, ExtraAvidin® and BSA (Fig 5.2B). These results suggest that HRG can bind directly and specifically to heparanase. According to Figure 5.1C above, proteolytically cleaved forms of HRG were found to co-purify with platelet-derived heparanase and may form a stronger interaction with heparanase than intact HRG. Since plasmin can efficiently cleave HRG at specific sites and generate proteolytic fragments of HRG that are of similar molecular weight to those observed in Figure 5.1C (see Chapter 4), the effect of plasmin cleavage on the ability of HRG to bind heparanase was also investigated. HRG\textsuperscript{PID} was subjected to plasmin treatment for 0, 30, 60 and 120 mins as described in Figure 4.2 to generate different proteolytically cleaved forms of HRG\textsuperscript{PID} prior to heparanase binding. Plasmin cleavage had no apparent effect on HRG\textsuperscript{PID} binding to heparanase immobilized on ELISA wells (Fig. 5.2C). These data suggest that the binding of HRG to heparanase is not modulated by plasmin-mediated cleavage and implies that the presence of HRG fragments in the heparanase preparation (see Fig. 5.1B) is probably a result of HRG degradation during the purification of platelet-derived heparanase.

To further investigate the interaction between HRG and heparanase, ELISA-based binding assays were performed under conditions that are known to modify the ligand binding capacity of HRG or were carried out in the presence of other HRG ligands. HRG\textsuperscript{PID} binding to heparanase was enhanced by acidic pH and inhibited by basic pH (Fig. 5.3A), whereas the presence of physiological concentrations of Zn\textsuperscript{2+} had no major effect on the heparanase-HRG interaction (Fig. 5.3B). These results indicate that the binding of HRG to heparanase is pH but not Zn\textsuperscript{2+} sensitive and that a change in the conformation and/or the net charge of HRG at different pHs may play an important role in regulating heparanase binding. Furthermore, ligands that are known to bind to the HRR of HRG, such as hemin, HS and heparin, markedly inhibited the interaction between HRG and heparanase (Fig. 5.3C, D and E), suggesting the potential involvement of the HRR of HRG in heparanase binding. Since the N1N2 domain of HRG has also been implicated in HS and heparin binding (Jones et al., 2004b), the
Figure 5.1: Absorbance of HRGβ (405 nm) with different concentrations of Hemin, HS, Heparin, PI-88, Dermatan sulfate, and Chondroitin sulfate A.

A: Absorbance of HRGβ (405 nm) with different pH values.

B: Absorbance of HRGβ (405 nm) with different concentrations of Zn2+.

C: Absorbance of HRGβ (405 nm) with different concentrations of Hemin.

D: Absorbance of HRGβ (405 nm) with different concentrations of HS.

E: Absorbance of HRGβ (405 nm) with different concentrations of Heparin.

F: Absorbance of HRGβ (405 nm) with different concentrations of PI-88.

G: Absorbance of HRGβ (405 nm) with different concentrations of Dermatan sulfate.

H: Absorbance of HRGβ (405 nm) with different concentrations of Chondroitin sulfate A.
Figure 5.3. Effect of pH, Zn$^{2+}$ and various HRG ligands on the interaction of HRG$^{PID}$ with heparanase. Analysis of the ability of HRG$^{PID}$ to bind heparanase (A) at different pH or (B) in the presence of Zn$^{2+}$ by ELISA using wells pre-coated with 2 $\mu$g/ml of heparanase. Error bars represent the range of duplicate samples. Effect of different concentrations of (C) hemin, (D) HS, (E) heparin, (F) PI-88, (G) dermatan sulfate, (H) chondroitin sulfate A, (I) cardiolipin, (J) human plasminogen and (K) normal human IgG on HRG$^{PID}$ (5 $\mu$g/ml) binding to heparanase was also examined by ELISA, with ELISA wells being pre-coated with 2 $\mu$g/ml of heparanase and then analyzed for HRG$^{PID}$ binding under the different conditions at pH 7.2. Error bars represent SEM (n = 3).
inhibitory effect of heparin and HS observed in Figure 5.3D and E may also indicate a possible role for the N1N2 domain of HRG in heparanase binding. In additional experiments, it was found that the sulfated oligosaccharide and anti-cancer drug PI-88 (containing a mixture of sulfated penta- and tetra-saccharides of mannose) also markedly inhibited HRG binding to heparanase (Fig. 5.3F). In contrast, dermatan sulfate and chondroitin sulfate A, sulfated glycosaminoglycans that are relatively poor HRG ligands (Olsen et al., 1996), had only a minor or no apparent effect on the interaction between HRG and heparanase (Fig. 5.3G and H). The recently identified phospholipid ligand of HRG, cardiolipin (see Section 3.3.12.3), also markedly reduced the binding of HRG to heparanase (Fig. 5.3I). In contrast, HRG ligands that are known to bind to the N- and C-terminal domains of HRG, such as human plasminogen and normal human IgG, had no apparent effect on HRG binding to heparanase (Fig. 5.3J and K). Collectively, these data suggest that the binding of some but not all HRG ligands can interfere with the interaction between HRG and heparanase, and ligands such as heparin, HS, hemin and phospholipids may play an important physiological role in regulating the binding of HRG to heparanase.

5.3.3 HRG\textsuperscript{PID} partially inhibits heparanase binding to cell surface receptors

Although the earlier sections of this chapter have clearly shown that HRG interacts with heparanase, the consequence of this interaction on the functions of heparanase needed to be determined. The ability of extracellular heparanase to tether onto cell surface HSPG and other receptor(s) such as LRP and the CIMPR is key to its activation, function and uptake into intracellular compartments (Nadav et al., 2002; Gingis-Velitski et al., 2004b; Zetser et al., 2004; Vreys et al., 2005; Ben-Zaken et al., 2007; Wood and Hulett, 2008). Since HRG has been proposed to act as an adaptor molecule and tether plasminogen to cell surfaces (Jones et al., 2004a), the ability of HRG to effect heparanase binding to the cell surface was investigated. Mock transfected mouse L cells (MS cells) and mouse L cells stably transfected with the 300 kDa CIMPR (MS9-II cells) were used, respectively, to examine heparanase binding to HSPG or to both HSPG and the CIMPR as described in a previous study (Wood and Hulett, 2008). Firstly, the cell surface
expression of the CIMPR and HS on MS and MS9-II cells was verified by immunofluorescence flow cytometry, the CIMPR being shown to be exclusively expressed on MS9-II cells (Fig. 5.4A), whereas both MS and MS9-II cells expressed a similar level of HS (Fig. 5.4B). Furthermore, Alexa-594 labelled HRG^{PID} (100 μg/ml) bound MS and MS9-II cells to a similar extent (Fig. 5.4C), possibly via cell surface HS. Consistent with previous studies by Wood and Hulett (2008), higher levels of biotinylated heparanase (4 μg/ml) bound to MS9-II than MS cells as a result of CIMPR expression on the MS9-II cells (Fig. 5.4D). Interestingly, pre-coating cells with HRG^{PID} (100 μg/ml) moderately (~23%) enhanced biotinylated heparanase (4 μg/ml) binding to MS cells, whereas no enhanced binding was observed with the MS9-II cells (Fig. 5.4D). These data indicate that surface bound HRG may enhance heparanase binding when a limited amount of heparanase receptors (e.g. the CIMPR) are expressed on the cell surface. In contrast, when cells were incubated simultaneously with both HRG^{PID} (100 μg/ml) and biotinylated heparanase (4 μg/ml), a partial reduction in biotinylated heparanase binding to MS and MS9-II cells was observed, i.e. ~33% and ~36%, respectively (Fig. 5.4D). These results suggest that under normal physiological conditions when both surface bound and soluble HRG are present, soluble HRG is likely to prevent heparanase binding to cell surface receptors, possibly via direct interaction with heparanase. It is also important to note that based on the data in Figure 5.4D, HRG may partially block heparanase binding to cell surface HSPG but it is uncertain whether HRG can inhibit heparanase binding to the CIMPR.

5.3.4 HRG^{PID} can directly enhance heparanase enzymatic activity

It has been reported previously that HRG can bind to heparanase-sensitive areas on HS and can potentially protect HS from heparanase cleavage (Freeman and Parish, 1997). Since HRG is the second most abundant plasma protein that can bind HS and heparin with high affinity (Heimburger et al., 1972; Lijnen et al., 1983), the ability of HRG to mask heparanase-sensitive sites on HS may play an important role in regulating heparanase functions. Thus, the effect of HRG on heparanase activity, as measured by
Figure 5.4. Effect of HRG$^{PD}$ on heparanase binding to cell surface receptors. (A) Cell surface expression of human CIMPR by mouse L cells stably transfected with human CIMPR (MS9-II cells) as detected by flow cytometry using a mouse anti-human CIMPR mAb (MEM-328) and a PE-conjugated sheep F(ab')$_2$ anti-mouse Ig Ab, with mock transfected mouse L cells (MS cells) being included as a negative control. Representative flow cytometry histograms are shown, with open blue histograms representing primary and secondary Ab only control, and open purple and filled green histograms representing cell surface expression of human CIMPR by MS and MS9-II cells, respectively. (B) Cell surface expression of HS by MS and MS9-II cells as detected by flow cytometry using a mouse anti-HS mAb (F58-10E4) and a FITC-conjugated sheep F(ab')$_2$ anti-mouse Ig Ab. (C) Ability of Alexa-594 labelled HRG (100 μg/ml) to bind to MS and MS9-II cells at pH 7.2 as detected by flow cytometry. (D) Ability of biotinylated heparanase (4 μg/ml) to bind to MS and MS9-II cells at pH 7.2 either in the presence of HRG$^{PD}$ (100 μg/ml) or to cells pre-coated with HRG$^{PD}$ (100 μg/ml). Data in (B), (C) and (D) are expressed as fold binding above background MFI, with error bars representing SEM (n = 3). NS, not significant. **, $P < 0.01$. ***, $P < 0.001$. 
the cleavage of \(^{3}\text{H}\)-HS, was investigated. Unexpectedly, the presence of HRG\(^{\text{PID}}\) markedly enhanced the cleavage of \(^{3}\text{H}\)-HS by heparanase in a concentration dependent manner under conditions where heparanase activity is high (i.e. pH 5.1) as well as low (i.e. pH 7.2) (Fig. 5.5A and B). The ability of HRG\(^{\text{PID}}\) to potentiate heparanase activity was not simply a result of an increase in protein concentration stabilizing the enzymatic activity of heparanase, as increasing concentrations of the control proteins OVA and a human IgG\(\kappa\) myeloma had little or no effect on heparanase enzymatic activity (Fig. 5.5A and B). To further verify that the ability of HRG\(^{\text{PID}}\) to enhance heparanase activity was not caused by the presence of heparanase in the HRG\(^{\text{PID}}\) preparation, HRG\(^{\text{PID}}\) alone showed no detectable heparanase activity at pH 5.1 (Fig. 5.5C). Collectively, these data suggest that HRG can regulate the function of heparanase by enhancing heparanase activity, probably by directly interacting with the enzyme.

5.4 Discussion

Heparanase is the only mammalian enzyme identified to date that is capable of cleaving HS and has been implicated in a variety of normal physiological processes such as embryonic development (Goldshmidt et al., 2001), wound healing (Zcharia et al., 2005) and angiogenesis (Elkin et al., 2001; Zcharia et al., 2005). In addition, elevated expression of heparanase has been associated with a number of pathological settings, including transformed tissues (see Vreys and David (2007) for review) as well as patients with kidney disorders (Katz et al., 2002; Maxhimer et al., 2005). Thus, the regulation of heparanase expression and function has been the subject of intensive research in recent years. The results presented in this chapter identify and characterize a novel mechanism for the regulation of heparanase function via the plasma glycoprotein HRG which can potentially modulate the role of heparanase in health and disease (summarized in Fig. 5.6).

HRG, an abundant multi-functional protein, is present in the plasma of many vertebrates and has a modular domain structure that can bind to a variety of ligands such as heparin,
Figure 5.5. HRG\textsuperscript{PID} enhances heparanase activity. Effect of HRG\textsuperscript{PID} on heparanase activity at (A) pH 5.1 (30 mins at 37°C) or (B) pH 7.2 (60 mins at 37°C), with OVA and a human IgG1κ myeloma being included as negative controls. Heparanase activity was measured by the cetylpyridium-chloride (CPC)-based [\textsuperscript{3}H]-HS precipitation assay as described in Section 2.24, with data being expressed as cpm/min ((Sample cpm – Blank cpm)/Incubation time). Error bars represent the range of duplicate samples. (C) Further confirmation of the absence of heparanase activity in a HRG\textsuperscript{PID} preparation at pH 5.1 for 30 mins at 37°C. Data are expressed as raw cpm values, with error bars representing the range of duplicate samples.
Figure 5.6. Regulation of heparanase functions by HRG. (1) Heparanase can be tethered to the cell surface and become internalized via cell surface heparan sulfate proteoglycan (HSPG) and the cation-independent mannose-6-phosphate receptor (CIMPR). (2) HRG binds heparanase and (3) partially inhibits heparanase binding to cell surface receptors. (4) HRG enhances heparanase activity and may play an important role in facilitating heparan sulfate (HS) cleavage to regulate various biological processes. (5) Soluble heparin and potentially cleaved HS fragments may in turn inhibit the interaction between HRG and heparanase.

HS, fibrinogen, plasminogen, C1q, IgG, Zn^{2+} and haem (Jones et al., 2005a). Furthermore, it has been suggested that HRG can regulate numerous biological processes such as immune complex/necrotic cell/pathogen clearance, angiogenesis, coagulation and fibrinolysis (see Section 1.1). In the current study, HRG was found to co-purify with platelet-derived heparanase (Fig. 5.1B and C) and a direct interaction between HRG and heparanase was also confirmed using an ELISA-based binding approach (Fig. 5.2A and B), indicating that heparanase can bind to other endogenous soluble proteins besides MBP. Surprisingly, HRG and MBP appear to have opposing effects on heparanase enzymatic activity. MBP was reported to be the key component in eosinophil lysates that can markedly inhibit the activity of heparanase (Temkin et al.,
In contrast, HRG enhances heparanase activity, both under neutral and acidic conditions (Fig. 5.5A and B). Most interestingly, both HRG and MBP can also bind to the heparanase substrate HS (Jones et al., 2004b; Swaminathan et al., 2005). Thus, the three-way interactions between heparanase, HS and HRG or MBP may provide novel mechanisms for regulating the enzymatic activity of heparanase. Unfortunately, due to the complex interaction between HRG, heparanase and heparanase-sensitive sites on HS (Fig. 5.2; Freeman and Parish, 1997), as well as the ability of HS to inhibit the interaction between HRG and heparanase (Fig. 5.3D), it is difficult to elucidate the molecular mechanisms underpinning the enhanced heparanase activity induced by HRG. Nevertheless, it is interesting to note that anti-heparanase antibodies have been shown to directly modify heparanase activity (Zetser et al., 2004; Gingis-Velitski et al., 2007). Gingis-Velitski et al. (2007) demonstrated that a mouse anti-heparanase mAb (6F8), which detects the C-terminus of heparanase, can enhance heparanase activity. Since the anti-heparanase mAb (6F8) recognizes an epitope that is not located at the predicted HS binding sites (Levy-Adam et al., 2005) or the active site of the molecule (Hulett et al., 2000), it was proposed that the mAb (6F8) may function by stabilizing an active conformation of heparanase (Gingis-Velitski et al., 2007). In contrast, a rabbit polyclonal anti-heparanase Ab (733), which recognizes the HS binding domain at the N-terminus of heparanase, inhibits heparanase activity (Zetser et al., 2004). Therefore, natural heparanase binding proteins such as HRG and MBP may interact with different domains of heparanase and differentially regulate heparanase enzymatic activity via analogous mechanisms to the anti-heparanase antibodies.

Besides regulating heparanase enzymatic activity, the current study also demonstrated that HRG can partially inhibit heparanase binding to cell surface receptors such as HSPG and possibly the CIMPR (Fig. 5.4D). Therefore, the presence of HRG at the relatively high concentration of ~100-150 μg/ml in human plasma is likely to have a major impact on the activation and function of heparanase by modulating the binding of heparanase to cell surfaces and the ECM. In addition, the binding of HRG to HS on cell surfaces and the ECM may result in HRG being localized to the microenvironment where heparanase activity is most likely to be present. Thus, HRG may aid heparanase
disassociation from cell surface receptors at the contact point between invading cells (e.g. T cells or metastatic tumor cells) and the ECM, and maintain heparanase in a soluble extracellular form available to degrade the ECM by preventing heparanase binding and internalization by surrounding cells. Furthermore, the direct interaction between HRG and heparanase (Fig. 5.2A and B), as well as the inhibitory effect of HRG on heparanase binding to cell surfaces (Fig. 5.4D), may also regulate non-enzymatic activities of heparanase, such as the ability to enhance cell adhesion (Goldshmidt et al., 2003) and directly stimulate intracellular signalling (Gingis-Velitski et al., 2004a). It is also worth noting that the binding of HRG and heparanase to cell surfaces is not mutually exclusive as a significant level of heparanase binding to cell surfaces was observed even in the presence of HRG (Fig. 5.4D) and vice versa (data not shown), suggesting that both HRG and heparanase can localize simultaneously on the cell surface. Interestingly, as described for heparanase, a previous study has reported that HRG can be internalized into intracellular compartments (Olsen et al., 1996), possibly via a HSPG-dependent mechanism (data not shown). Thus, HRG and heparanase can potentially be internalized into the same intracellular compartment, in which HRG may regulate heparanase activity and function intracellularly.

In addition, the experimental data presented in this chapter demonstrate that the interaction between HRG and heparanase can be regulated by a number of factors such as pH (Fig. 5.3A), indicating that HRG may modulate heparanase function in a pH-dependent manner, e.g. at sites of tissue injury where the local pH decreases (Punna-Moorthy, 1987; LaManna, 1996). Paradoxically, HRG was equally effective in enhancing heparanase activity at both neutral and acidic pH (Fig. 5.5A and B), although multiple interactions between heparanase, HS and HRG presumably affected heparanase enzymatic activity. Interestingly, hemin, HS as well as the anionic phospholipid cardiolipin efficiently inhibited the binding of HRG to heparanase (Fig. 5.3C, D and I), suggesting that the release of hemin/haem following hemolysis (Ascenzi et al., 2005), the exposure of anionic phospholipids at sites of coagulation (Shaw et al., 2007) and cell death (see Chapter 3) or the release of HS fragments following heparanase cleavage (Elkin et al., 2001; Li et al., 2005) may in turn abolish the ability of HRG to regulate the
function of heparanase. Furthermore, other abundant plasma ligands of HRG such as plasminogen and IgG had no apparent effect on HRG binding to heparanase (Fig. 5.3J and K), indicating that HRG can potentially act as an adaptor molecule between these proteins and that the presence of these HRG ligands in plasma is unlikely to hinder the interaction between HRG and heparanase. Lastly, the presence of the anti-coagulant heparin and the anti-cancer drug PI-88 markedly reduced the binding of HRG to heparanase (Fig. 5.3E and F). Thus, the release of heparin following mast cell degranulation (Lindstedt and Kovanen, 2006) or administration of heparin and PI-88 clinically may alter the function of HRG, in particular the ability of HRG to regulate the function of heparanase.

Collectively, the results presented in this chapter describe a novel mechanism for the regulation of heparanase function via the plasma protein HRG and represent the first identification of an endogenous ligand that enhances heparanase activity. The biological significance of the interaction between heparanase and HRG awaits further investigation and it will be of particular interest to determine how HRG may influence various normal physiological and pathological roles of heparanase such as HS turnover, wound healing, angiogenesis and cancer metastasis.
Chapter Six

FINAL DISCUSSION

This chapter provides a summary and a general discussion of the key findings described in Chapter 3, 4 and 5 of this thesis.
6.1 Introduction

HRG is a multi-functional adaptor protein that can interact with a variety of molecules including IgG, C1q, heparin, HS, plasminogen, fibrinogen, TSP, haem and Zn\(^{2+}\). Thus, HRG has been implicated in numerous biological processes, in particular immune complex/necrotic cell/pathogen clearance, cell adhesion, angiogenesis, coagulation and fibrinolysis. The experimental data presented in this thesis further support the role of HRG in immunity and vascular biology by elucidating the underpinning molecular mechanisms. The results reported in Chapter 3 of this thesis demonstrate that a complex consisting of both HRG and IgG is required to aid the removal of necrotic cells by monocytes via a FcγRI- and HS-dependent mechanism. In addition, Chapter 3 describes a role for intracellular phospholipids and phosphorylated proteins in mediating HRG binding to necrotic cells as well as stimulating the release of pro-inflammatory cytokines, such as IL-8 and TNF, following HRG-IgG complex enhanced necrotic cell uptake. Furthermore, the data presented in Chapter 4 suggest a model in which the function of HRG is regulated by plasmin-mediated proteolytic cleavage and provide a potential feedback mechanism for regulating the plasminogen/plasmin system. Finally, Chapter 5 describes a novel interaction between HRG and the HS-degrading enzyme heparanase, in which the presence of HRG can modulate heparanase enzymatic activity and the binding of heparanase to cell surfaces. Thus, this final chapter discusses these findings in the context of the literature, and explores future research directions made possible by this study.

6.2 HRG functions as an innate molecule that aids the removal of necrotic cells and pathogens

Numerous pattern recognition molecules (PRMs), such as TLRs, CRP, C1q and MBL, have been shown to play an important role in detecting and eliminating foreign materials/pathogens as well as dying/dead host cells (Stuart and Ezekowitz, 2005) (see Section 1.2). However, the molecular components that are involved in sensing and clearing necrotic cells are often poorly characterized. The results described in Chapter 3
of this thesis demonstrate that HRG may function as a PRM by recognizing anionic structures exposed in necrotic cells. Following binding, HRG can tether a specific subclass of human IgG, namely IgG2κ, to permeabilized/necrotic cells, whereby the IgG component can act as an adaptor molecule between the necrotic cell-bound HRG and FcγR on phagocytes (Fig. 6.1). Interestingly, recent studies have also reported that serum IgG from SLE patients, in contrast to serum IgG from healthy donors, can bind strongly to necrotic cells and modify the phagocytic pathways used to remove necrotic cells (Grossmayer et al., 2007; Reefman et al., 2007) (see Section 1.2.3.2). Therefore, as well as playing a fundamental role in the adaptive immune response against foreign antigens (Mix et al., 2006), IgG and FcγR can also modulate the clearance of dying/dead cells. Since a specific subclass of IgG and FcγR on phagocytes are key components in necrotic cell uptake enhanced by HRG (Chapter 3), it would be of particular interest to examine, in addition to HRG deficient (HRG−/−) mice, whether mice that lack serum IgG (e.g. B cell-deficient (μMT) mice) and mice that lack FcγR (e.g. FcγRI-deficient mice) or have impaired FcγR-dependent signaling (e.g. Fc receptor common γ-chain-deficient mice) show any impairment in sensing and eliminating dying/dead cells in vivo.

To the best of our knowledge, since all the HRGp preparations used in this thesis were purified from healthy donors, the experimental data presented in Chapter 3 suggest a model whereby healthy individuals may contain 'natural' antibodies, or simply a specific subclass of IgG, that can bind strongly to HRG and function co-operatively with HRG to facilitate necrotic cell clearance. Alternatively, the co-purified IgG present in the HRGp preparations may represent true autoantibodies generated as a result of autoimmunity. However, as HRG interacts directly with IgG (Gorgani et al., 1997; Gorgani et al., 1999c), it is unclear from the data presented in this thesis whether the co-purified IgG is indeed an autoantibody that specifically recognizes HRG via its antigen binding sites or normal IgG that binds HRG via another region. Nevertheless, further studies comparing the ability of purified IgG from a large cohort of healthy controls and patients with APS/SLE to bind to HRG may provide associative evidence to address whether high-affinity HRG-binding autoantibodies are present in clinically
Figure 6.1. The role of HRG in the clearance of necrotic cells and pathogens. HRG can potentially interact with a variety of opsonins, receptors and ligands to regulate the removal of permeabilized/necrotic cells and the subsequent immune response. HRG may also aid pathogen lysis and phagocytic uptake. CR3, complement receptor 3; DNA, deoxyribonucleic acid; FcγR, Fcγ receptor; HSPG, heparan sulfate proteoglycans; IC, immune complexes; Ig, immunoglobulin; TSP-1, thrombospondin-1.
healthy individuals or generated as a result of a systemic autoimmune response. Furthermore, since IgG2 was the main IgG subclass that co-purified with HRG, it would be of great interest to investigate whether IgG2 deficient patients, a deficiency known to increase the susceptibility of patients to encapsulated bacterial pathogens (Buckley, 2002) and associated with autoimmune diseases (Wilson et al., 1990; Eriksson et al., 1994), have antibodies that can interact strongly with HRG. The results from these studies may shed light on the role of HRG-IgG complexes in health and disease.

It is worth noting that the specific binding of the co-purified IgG to necrotic but not viable cells via HRG may have important implications for the normal physiological and pathological uptake of necrotic cells. For example, phagocytosis of HRG-IgG opsonized necrotic cells via a FcγRI-dependent mechanism may provide an efficient means of removing permeabilized cells to prevent further tissue damage, as well as recruiting additional leukocytes to sites of tissue injury via pro-inflammatory cytokines such as IL-8 and TNF. Indeed, binding of immune complexes (IC) to FcγRIIA on apoptotic neutrophils and subsequent clearance by macrophages via FcγR has been proposed to be an important mechanism for mediating the resolution of inflammation by efficiently removing the large number of neutrophils that are recruited to inflammatory sites and die locally via apoptosis (Hart et al., 2004). On the other hand, phagocytosis of necrotic cells by DCs via FcγR may aid the presentation of antigens derived from the permeabilized cells to autoreactive T cells in a pro-inflammatory manner and exacerbate autoimmunity. Therefore, it is of particular interest to examine whether HRG−/− mice are more or less prone to autoimmune disease.

Under physiological conditions, HRG may interact with other serum opsonins and modulate the uptake of necrotic cells via other phagocytic pathways besides the FcγR- and HS-dependent mechanism described in Chapter 3 (summarized in Fig. 6.1). HRG has been shown previously to bind C1q (Gorgani et al., 1997) as well as IC (Gorgani et al., 1999b; Gorgani et al., 1999a). Thus, necrotic cell-bound HRG may act as a platform to tether IC and C1q to the surface of necrotic cells and facilitate the activation of the classical complement pathway. Since complement activation has been shown to play a
critical role in apoptotic cell removal (Mevorach et al., 1998; Taylor et al., 2000; Ogden et al., 2001), HRG can potentially utilize the classical complement pathway to aid the uptake of necrotic cells. Interestingly, HRG could further modulate the complement-dependent phagocytic pathway by regulating the function of CR3, a key receptor for apoptotic cell uptake (Mevorach et al., 1998). Besides recognizing the complement fragment C3bi following complement activation, CR3 has been shown previously to bind fibrinogen and this interaction can subsequently block the binding of C3bi to CR3 (Wright et al., 1988). The ability of HRG to bind fibrinogen (Leung, 1986) may lead to the formation of an adaptor complex that can link necrotic cells to CR3 on phagocytes. Alternatively, the interaction between HRG and fibrinogen may inhibit the binding of fibrinogen to CR3 and increase the availability of CR3 to recognize C3bi opsonized dying/dead cells. In addition, HRG has been reported to bind TSP-1 (Leung et al., 1984; Walz et al., 1987), a multi-functional protein that can act as a bridging molecule between dying/dead cells and CD36 and αvβ3 integrin on macrophages (Savill et al., 1992; Ren et al., 2001). Since HRG contains two CLESH-1 motifs that resemble the TSP-binding motif on CD36 (Simantov et al., 2001; Simantov et al., 2005), HRG may interfere with TSP-1-CD36-mediated clearance of dying/dead cells. Taken together, HRG can potentially regulate multiple different phagocytic pathways and depending on the type of phagocytic cell (e.g. DC, monocyte, macrophage or neutrophil), the immunological consequences of necrotic cell uptake enhanced by HRG may also vary due to the differential expression of various phagocytic receptors, such as CR3 and FcγR, by different phagocytes (van de Winkel and Anderson, 1991; Bajtay et al., 2006).

Besides the removal of necrotic cells, HRG has also been shown to act as a defence molecule against pathogens such as Enterococcus faecalis, Escherichia coli and Candida albicans (Kacprzyk et al., 2007; Rydengard et al., 2007; Rydengard et al., 2008). Although HRG and peptides derived from the HRR of HRG can mediate the direct killing of pathogens by triggering membrane destabilization (Kacprzyk et al., 2007; Rydengard et al., 2007; Rydengard et al., 2008), HRG may also aid the uptake of pathogens via a similar mechanism to necrotic cell clearance. Indeed, preliminary data suggest that HRG-IgG complexes in the HRG preparation can bind to Escherichia coli.
Interestingly, similar to other PRMs such as MBL and C1q, the results presented in Chapter 3 demonstrate that HRG can bind to a broad range of molecules including HS, cardiolipin, phosphatidic acid (PA), lysophosphatidic acid, sulfatide, phosphatidylinositol (PtdIns), PtdIns(4)P, PtdIns(5)P, PtdIns(4,5)P2, PtdIns(3,5)P2 and possibly phosphorylated proteins. Thus, HRG may function as a PRM that recognizes specific molecular pattern exposed on necrotic cells and pathogens. Furthermore, the ability of heparin to block HRG binding to these cellular molecules (see Chapter 3) and pathogens (Rydengard et al., 2007; Rydengard et al., 2008) indicates that the heparin-binding domains on HRG (i.e. the HRR and N1N2 domain) may overlap with its pattern recognition region(s). Although the detailed molecular basis of the interaction between HRG and pathogens is currently unknown, the ability of HRG to interact with various negatively charged molecules, as described in Chapter 3, suggests that the pathogen ligand(s) for HRG is likely to be anionic in nature. Collectively, similar to activation of the complement pathways (see Section 1.2.3.1), HRG may have multiple roles in the defence against invading pathogens by promoting cell lysis and phagocytic uptake of pathogens, as well as initiating a pro-inflammatory response to recruit leukocytes to sites of infection. Indeed, the role of HRG in pathogen clearance was demonstrated recently using HRG−/− mice, in which HRG−/− mice were substantially more susceptible to Candida albicans infection than wild type C57BL/6 mice (Rydengard et al., 2008).

6.3 HRG regulates the function of matrix degrading enzymes

Matrix degradative enzymes like plasmin, matrix metalloproteases (MMPs), urokinase plasminogen activator (u-PA) and heparanase are key mediators of various physiological processes such as angiogenesis, inflammation and wound healing. However, the function of these enzymes is often dysregulated in pathological conditions such as cancer metastasis, chronic inflammation and tissue destruction (Duffy, 1996; Castellino and Ploplis, 2005; Dano et al., 2005; Ra and Parks, 2007; Vreys and David, 2007; Nasser, 2008). Thus, the catalytic function of extracellular degradative enzymes is tightly regulated by a variety of molecular mechanisms to ensure precise activation of enzymatic activity and prevent unnecessary tissue damage. In general, degradative
enzymes can be regulated at six points, namely at the level of (1) gene expression, (2) localization/compartimentalization, (3) zymogen/pro-enzyme activation, (4) enzyme inactivation, (5) availability of substrates and (6) enzymatic activity (Ra and Parks, 2007). The experimental data presented in Chapters 4 and 5 demonstrate that HRG may play a vital role in regulating the function of the serine protease, plasmin, and the endo-\(\beta\)-D-glucuronidase, heparanase, by various mechanisms (discussed below).

Although the ability of plasmin to cleave HRG as well as the interaction between HRG and plasminogen has been well documented in the literature (Lijnen et al., 1980; Smith et al., 1985; Saez et al., 1995; Borza et al., 1996; Borza and Morgan, 1997; Borza et al., 2004; Jones et al., 2004a), the potential feedback regulation of the plasminogen/plasmin system via plasmin-cleaved HRG has not been thoroughly investigated. The results reported in Chapter 4 demonstrate that the initial plasmin cleavage of HRG can effectively reduce HRG binding to cell surface HS by >50%, but cannot inhibit HRG binding to plasminogen, suggesting that plasmin cleavage may inhibit the ability of HRG to tether plasminogen to cell surfaces for activation and the plasmin-cleaved HRG may act as a decoy/clearance receptor for plasminogen. Interestingly, additional studies also showed that the presence of Zn\(^{2+}\), acidic pH or heparin, can protect HRG from plasmin cleavage, indicating that the tight binding of HRG to cell surface HS under conditions of tissue injury (i.e. low pH and elevated Zn\(^{2+}\)) may preserve the ability of HRG to function as an adaptor molecule to tether plasminogen to cell surfaces. Therefore, based on the evidence reported in Chapter 4 of this thesis and previous studies by Lijnen et al. (1980), Borza and Morgan (1997), Borza et al. (2004), Jones et al. (2004a) and Jones et al. (2004b), the following model for HRG regulation of the plasminogen/plasmin system is proposed (summarized in Fig. 6.2). During the initial phase of tissue injury/remodelling or wound healing, the presence of Zn\(^{2+}\) and acid pH favours HRG-mediated tethering of plasminogen to cell surfaces and HRG functioning as an activating receptor for plasminogen activation. However, when excessive amounts of plasmin are generated and/or the local pH and Zn\(^{2+}\) concentrations have returned to normal physiological levels, HRG is more prone to proteolytic cleavage by plasmin, a
Figure 6.2. Regulation of the plasminogen/plasmin system via HRG. (1) HRG binds plasminogen and (2) aids the tethering of plasminogen to the cell surface via heparan sulfate proteoglycans (HSPG). (3) Tethering plasminogen to the cell surface via HRG may enhance plasminogen activation to plasmin by urokinase-type or tissue-type plasminogen activators (u-PA or t-PA) bound to the cell surface via u-PA or t-PA receptors (u-PAR or t-PAR). (4) Plasmin can in turn mediate the proteolysis of HRG and reduce the ability of HRG to bind to cell surface HSPG. (5) Plasmin-cleaved HRG (PC-HRG) may act as a decoy receptor to limit further plasminogen activation. The potential ability of HRG/PC-HRG to modulate the interaction between plasminogen and other receptors/ligands is not depicted in the figure.

process that favours HRG acting as a decoy/clearance receptor for plasminogen and attenuates further plasminogen activation. In addition, once HRG has been cleaved by plasmin, it can no longer respond to environmental factors (e.g. pH and Zn\(^{2+}\)) that are known to potentiate its ability to tether plasminogen to cell surfaces, making plasmin-cleaved HRG an ideal decoy/clearance receptor.
Besides regulating the plasmin/plasminogen system, the experimental data presented in Chapter 5 demonstrate that HRG can also interact directly with heparanase and modulate heparanase function by blocking heparanase binding to cell surface receptors and enhancing heparanase enzymatic activity in solution. Although these initial results suggest that HRG may play a role in regulating heparanase function during normal physiological processes (e.g. embryonic development, HS turnover, angiogenesis, inflammation and wound healing) as well as during pathological conditions (e.g. tumorogenesis and kidney disease), further functional studies (e.g. ECM degradation assays) are necessary to address whether HRG is a positive or a negative regulator of heparanase function. In addition, the molecular mechanisms underpinning the ability of HRG to enhance heparanase activity require further investigation to elucidate whether HRG can increase the presentation of the HS substrate to heparanase or simply stabilize an enzymatically active conformation of heparanase. It is equally important to identify the binding sites on both HRG and heparanase that mediate their interaction, possibly by using peptides derived from different domains of HRG and heparanase as blocking agents in binding assays, or by generating recombinant fragments of HRG and heparanase to examine their ability to directly interact.

In addition to the above-mentioned mechanisms, the ability of HRG to modulate the function of both heparanase and the plasminogen/plasmin system may generate a complex network of regulatory pathways that not only control the role of HRG in various biological processes, but also aid cross-talk between these two classes of degradative enzymes. For example, since the function of HRG is often dependent on HS/heparin binding, such as the phagocytic uptake of necrotic cells (Chapter 3) and the tethering of plasminogen to GAG surfaces (Borza et al., 2004; Jones et al., 2004a) (Chapter 4), regulating heparanase-mediated HS cleavage via HRG (Chapter 5) may in turn alter the ability of HRG to mediate necrotic cell clearance as well as plasminogen activation. Furthermore, the reduced ability of HRG to bind to HS following plasmin-mediated cleavage (Chapter 4) may abolish the potential role of HRG in masking heparanase-sensitive areas on HS (Freeman and Parish, 1997), which could increase the availability of HS for heparanase binding and cleavage.
It is also worth noting that HRG can potentially indirectly regulate the expression of degradative enzymes through modulating the biological activity of basic fibroblast growth factor (bFGF). bFGF has been shown to induce the expression of MMP-13 (collagenase-3), an important protease that mediates the degradation of collagen and the proteoglycan molecule aggrecan (Im et al., 2007; Muddasani et al., 2007). Similarly, bFGF can also stimulate the expression of u-PA, a key protease that catalyzes the conversion of plasminogen to plasmin (Quarto and Amalric, 1994; Besser et al., 1995; Roghani et al., 1996). Thus, the ability of HRG to regulate the availability of bFGF to signal through HSPG and FGF receptors (Brown and Parish, 1994) may in turn alter the expression of MMP-13 and u-PA.

6.4 Lessons to learn from HRG and other functionally related plasma proteins

Certain plasma proteins are essential for a variety of normal physiological processes, such as coagulation, fibrinolysis, angiogenesis, tissue remodelling, transport of nutrients as well as the detection and clearance of unwanted materials like pathogens, immune complexes and dying/dead cells. Interestingly, a number of well characterized multi-functional plasma proteins involved in innate immunity and tissue repair, including HMWK, β₂GPI, CRP, SAP, Clq, MBL and TSP-1, appear to share the same functional properties as HRG (see Table 6.1). Although it is not surprising that proteins belonging to the same superfamily (e.g. CRP and SAP are characterized as pentraxins, whereas HRG and HMWK are members of the cystatin supergene-family) have similar biological properties, it is becoming clear from the current literature and results described in this thesis that HRG shares many functional similarities with other structurally unrelated plasma proteins (Table 6.1).

Although HRG and the functionally related serum proteins listed in Table 6.1 have not been shown to share any apparent sequence homology with each other, unless they are from the same superfamily, most of these proteins have been reported to bind to the same endogenous ligands, in particular negatively charged molecules such as heparin,
Table 6.1. Functional similarities between HRG and other plasma proteins.

<table>
<thead>
<tr>
<th>Function</th>
<th>HRG</th>
<th>CRP</th>
<th>MBL</th>
<th>HMWK</th>
<th>C1q</th>
<th>C2/C4/C3</th>
<th>C1r</th>
<th>TSP-1</th>
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<tbody>
<tr>
<td>Regulated by divalent ions</td>
<td>Zn(^{2+})</td>
<td>Zn(^{2+})</td>
<td>Ca(^{2+})</td>
<td>Ca(^{2+})</td>
<td>Ca(^{2+})</td>
<td>Ca(^{2+})</td>
<td>Ca(^{2+})</td>
<td>Ca(^{2+})</td>
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<tr>
<td>Regulated by plasmin cleavage</td>
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<tr>
<td>Regulates angiogenesis</td>
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<tr>
<td>Function that has not been reported in the literature</td>
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**Notes:**
- CRP, C-reactive protein; HMWK, high-molecular-weight kininogen; MBL, mannose-binding lectin; C1r, C1r; TSP-1, tissue-type plasminogen activator.
anionic phospholipids and DNA. Whether these serum proteins share a similar protein
domain or simply possess positively charged regions which mediate ligand binding
requires further investigation. Nevertheless, their ability to interact with negatively
charged molecules may facilitate their interaction with various pathogens, such as
Helicobacter pylori (Smith et al., 1990), Vibrio cholerae (Kabir and Ali, 1983),
Escherichia coli (Sherman et al., 1987) and the influenza virus (Huang, 1974), which
have been shown to possess negatively charged surfaces. Importantly, the ability of
multiple different plasma proteins to recognize a broad range of negatively charged
molecules on pathogens may provide an effective mean of clearing pathogens via
different molecular mechanisms (e.g. complement- versus non-complement-based
mechanisms) and thus prevent immune evasion by pathogens.

Furthermore, the ability of these functionally related proteins to interact with various
phospholipids may play a vital role in dying/dead cell clearance, whereby the exposure
of anionic phospholipids is a common characteristic of most apoptotic (Balasubramanian
and Schroit, 2003) and necrotic cells (Hirt and Leist, 2003; Brouckaert et al., 2004;
Bottcher et al., 2006). Indeed, most of the proteins listed in Table 6.1 have been reported
to aid the recognition and removal of dying/dead cells (see Section 1.2). Interestingly,
the ability of β2GPI to bind to various phospholipids, especially to phospholipids
exposed on dying/dead cells, has been proposed to trigger the generation of
autoantibodies to β2GPI and β2GPI-phospholipid complexes (Levine et al., 1998).
Therefore, the production of autoantibodies against plasma proteins such as HMWK
(Sugi and McIntyre, 1995), CRP (Sjowall and Wettero, 2007) and possibly HRG
(Chapter 3), could be initiated by the interaction between these functionally related
proteins and phospholipids exposed on dying/dead cells. It would be of great interest in
future studies to examine whether immunization of HRGΔmice with HRG-coated
necrotic cells or simply challenging wild type mice with necrotic cells could induce the
production of autoantibodies against HRG. Although the presence of autoantibodies
against various plasma proteins can potentially lead to the onset and maintenance of
pathological conditions (e.g. by blocking the normal function of the targeted protein,
generating excess amounts of immune complexes in the circulation or depositing
autoantibodies on healthy tissues), tethering antibodies to pathogens via various opsonins may be beneficial during infections. Interestingly, IgG autoantibodies against C1q (Haseley et al., 1997; Norsworthy et al., 1999), β2GPI (Arvieux et al., 1994) and possibly HRG (Chapter 3) are often skewed toward the IgG2 subclass, the IgG subclass that is associated with ‘natural’ antibodies that recognize carbohydrate structures on encapsulated bacteria (Buckley, 2002). Thus, the antibodies that recognize various plasma opsonins may represent ‘natural’ antibodies, which could provide an evolutionary advantage by forming a complex with plasma opsonins which detect a broad range of molecular patterns exposed on pathogens or dying/dead cells and subsequently aid their removal via a FcγR-dependent mechanism.

As mentioned above (also see Section 1.2.2), phospholipid asymmetry on the plasma membrane of dying/dead cells plays an important role in exposing ‘eat-me’ signals, such as PS, to trigger phagocytic uptake (Balasubramanian and Schroit, 2003). The results presented in Chapter 3 of this thesis suggest that when the cell membrane has become permeabilized, intracellular phospholipids like PtdIns(4)P and PA are also exposed as additional ‘eat-me’ signals to enhance necrotic cell clearance via the plasma opsonin HRG. In order to determine whether PtdIns(4)P and PA are indeed novel ‘eat-me’ signals for necrotic cell disposal, it is essential to first quantify the exposure of these intracellular phospholipids on different cell types during different stages of cell death, as well as on cells that have been induced to undergo cell death via different stimuli. Furthermore, since most of the functionally related proteins listed in Table 6.1 can interact with various phospholipids (e.g. cardiolipin, PE and PS) and have been proposed to specifically recognize permeabilized/necrotic cells (see Section 1.2.3), it is of particular interest to investigate whether these plasma opsonins can also bind to intracellular phospholipids like PtdIns(4)P and PA. Besides binding negatively charged phospholipids, the results reported in this thesis also demonstrated that HRG can potentially interact with phosphorylated proteins exposed in necrotic cells, in particular phosphoserine containing proteins (Chapter 3). The ability of phosphorylated molecules to function as a novel signal to indicate the presence of permeabilized cells, as well as provide a possible ‘danger’ signal associated with necrotic cells, is an attractive
hypothesis since protein phosphorylation doesn’t usually occur extracellularly except during specific physiological processes such as neurite outgrowth, synapse/bone formation, myogenic differentiation and IgE-mediated mast cell degranulation (Redegeld et al., 1999). Thus, similar to a chemokine/cytokine gradient, the release of protein complexes containing phosphorylated proteins and kinases that may phosphorylate the local extracellular environment, can potentially generate a phosphorylated protein gradient which may aid the recruitment of leukocytes to the site of necrotic cell death. Since CRP (Culley et al., 1996; Culley et al., 2000) and SAP (Loveless et al., 1992) have also been shown to bind to phosphorylated carbohydrates, these functionally related proteins may play an important role in detecting the presence of necrotic cells via the recognition of phosphorylated molecules. Moreover, since HRG and other functionally related proteins may share a similar ligand specificity as intracellular signalling molecules (e.g. Shc) that also recognize both phosphorylated proteins and phospholipids (Zhou et al., 1995), further bioinformatic as well as structural studies would be of particular interest to examine whether various plasma proteins and intracellular signalling molecules contain any domain or structural similarities. In addition, pull-down experiments using, for example, PtdIns(4)P or PA-containing liposomes or phosphoserine/threonine/tyrosine-conjugated beads, may assist in the identification of other plasma proteins or cell surface receptors that can recognize these novel necrotic cell-associated ligands.

In addition to the clearance of pathogens and dying/dead cells, exposure of negatively charged surfaces may also aid the activation of the kallikrein-kinin system (KKS), which may play an important role in regulating coagulation at sites of tissue destruction or a developing thrombus, as well as other physiological processes including blood pressure and flow, cell proliferation, angiogenesis, apoptosis and inflammation (Schmaier and McCrae, 2007). The KKS consists of three so-called ‘contact factors’, namely the two zymogens, factor XII and prekallikrein, and the substrate/cofactor, HMWK (Schmaier and McCrae, 2007). Although the KKS was originally described as a surface-activated coagulation system that occurs on negatively charged surfaces, the assembly and activation of the KKS has also been shown to involve other cell surface molecules such
as globular heads of complement C1q (gC1qR), urokinase-type plasminogen activator receptor (u-PAR), cytokeratin 1 and tropomysin (Schmaier and McCrae, 2007). Interestingly, besides being identified as a member of the cystatin supergene-family (Koide, 1988), HRG seems to share some striking similarities with HMWK, namely the ability of HRG to bind negatively charged molecules (Chapter 3) and tropomysin (Donate et al., 2004), as well as the sensitivity of HRG to kallikrein-mediated proteolytic cleavage (Smith et al., 1985). Thus, whether HRG participates in the KKS and regulates various biological processes through the KKS warrants further investigation.

The ability of proteases to regulate the function of HRG (see Chapter 4) and other functionally related proteins (see Table 6.1) is another common feature that is shared by these plasma proteins. For example, a combination of plasmin- and kallikrein-mediated cleavage of HMWK may aid the generation of kinins (Kleniewski et al., 1992), which are potent vasodilator oligopeptides that can regulated blood pressure and inflammation (Campbell, 2000). Proteolytic cleavage of HMWK can also generate two-chain HMWK that inhibits angiogenesis (Zhang et al., 2000). Similarly, cleavage of β2GPI by plasmin (Sakai et al., 2007) and polymorphonuclear neutrophil (PMN)-derived proteases (Nilsson et al., 2008) has also been shown to regulate the ability of β2GPI to exhibit anti-angiogenic and anti-bacterial activities. Thus, the generation of active proteases (e.g. at sites of tissue injury and remodelling) may play a vital role in controlling the multi-functional activities of the functionally related proteins listed in Table 6.1. Since the proteolytic cleavage of these plasma proteins is often found to produce fragments with anti-angiogenic and anti-microbial activities, it would be of great interest to examine whether the cleaved fragments of these functionally related proteins exhibit overlapping or distinct properties.

6.5 Conclusion

HRG has been shown to participate in multiple normal physiological and pathological processes through its ability to interact with a variety of different ligands. However, the
molecular mechanisms underpinning the effect of HRG on many of these biological processes remain largely unknown. The experimental data presented in this thesis not only characterized in some detail the molecular components involved in enhanced necrotic cell uptake by HRG but also identified new HRG ligands, such as anionic phospholipids, sulfated glycosphingolipid, phosphorylated proteins and heparanase. In addition, studies of the proteolytic cleavage of human HRG by plasmin provided an additional mechanism for regulating the function of HRG at sites of tissue injury and remodelling. Collectively, these findings offer novel research avenues in the future to dissect the role of HRG in coagulation, autoimmunity, wound healing, angiogenesis and cancer progression.
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adaptor protein in plasma that modulates the immune, vascular and coagulation systems. 

specifically binds to necrotic cells via its amino-terminal domain and facilitates necrotic cell 

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