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Histidine-rich glycoprotein functions cooperatively with cell surface heparan sulfate on phagocytes to promote necrotic cell uptake

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ABSTRACT

Dying cells, such as apoptotic and necrotic cells, are cleared rapidly from the site of cell death to prevent the exposure of intracellular antigenic and immunostimulatory molecules that may cause tissue injury or facilitate the development of autoimmune diseases. For the immune system to recognize and remove dying cells efficiently, professional phagocytes use a variety of mechanisms that distinguish healthy cells from dying cells. HRG, a relatively abundant heparin/HS-binding protein in human plasma, has been shown recently to tether IgG specifically to necrotic cells and aid the phagocytic uptake of necrotic cells via a FcγRI-dependent pathway. In this study, we provide direct evidence that HRG can function cooperatively with cell surface HS on the monocytic cell line THP-1 to promote necrotic cell removal. In addition, we found that the presence of heparin can markedly inhibit HRG-enhanced necrotic cell clearance by THP-1 cells, possibly by blocking the ability of HRG to interact with necrotic cells as well as THP-1 cells. Thus, these data suggest that HRG can aid the phagocytosis of necrotic cells via a HS-dependent pathway, and this process can be regulated by the presence of certain HRG ligands, such as heparin.
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Introduction

During the process of development and wound healing, as well as to maintain tissue homeostasis, cells of a multicellular organism often die through the well-defined process known as programmed cell death or apoptosis. Early apoptotic cells are characterized as cells in which the plasma membrane remains intact but exposes the so-called "eat-me" signals on the cell surface, such as phosphatidylserine, to mediate recognition and phagocytic removal by phagocytes. If apoptotic cells persist as a result of a deficiency in clearance and/or an overload of dying cells, early apoptotic cells can become late apoptotic cells when the plasma membrane becomes permeabilized. Similarly, direct exposure of healthy, viable cells to trauma, such as extreme temperature or mechanical and chemical insults, can also result in the generation of membrane-permeabilized necrotic cells. Under normal physiological conditions, neighboring cells, as well as professional phagocytes, such as macrophages and dendritic cells, can efficiently detect and differentiate the different types of dying cells from viable cells via a number of mechanisms. In particular, professional phagocytes of the innate immune system often use a variety of germ-line-encoded receptors and opsonins to recognize and bind the dying cells, which can subsequently trigger the intracellular signaling events required for phagocytosis [1].

HRG, a member of the cystatin supergene family, is a relatively abundant, multifunctional protein that is present in the plasma of many vertebrates. The ability of HRG to interact simultaneously with a variety of plasma proteins as well as cell surface receptors has suggested that HRG can act as an adaptor molecule that regulates numerous biological processes, such as immune complex and pathogen clearance, cell adhesion, angiogenesis, coagulation, and fibrinolysis [2, 3]. In addition to these proposed functions, studies by Gorgani et al. [4] demonstrated that HRG binds preferentially to radiation-treated late apoptotic cells, possibly via direct interaction with naked DNA exposed during apoptosis. In the same study, it

Abbreviations: GAG=glycosaminoglycan, HRG=histidine-rich glycoprotein, HRG^{P}=plasma-derived histidine-rich glycoprotein, HRG^{PID}=plasma-derived IgG-depleted histidine-rich glycoprotein, HRR=histidine-rich region of histidine-rich glycoprotein, HS=heparan sulfate, HSPG=heparan sulfate proteoglycans, IgG^{HRG}=the copurified IgG isolated from plasma-derived histidine-rich glycoprotein, MFI=mean fluorescence intensity, N1N2=N-terminal domains of histidine-rich glycoprotein

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was also suggested that HRG can bind to FcγRI on human monocyte-derived macrophages and function as a bridging molecule to facilitate the removal of a mixture of early and late apoptotic cells via a FcγRI-dependent mechanism [4]. Similarly, later studies by Jones et al. [5] showed that HRG binds strongly to cytoplasmic ligand(s) exposed on membrane-permeabilized, heat-killed necrotic cells via the N-terminal domains (N1N2) and aids their phagocytic uptake by THP-1 cells (a human macrophage-like monocytic leukemia cell line). Interestingly, HRG has also been shown to facilitate complement activation on necrotic cells, possibly through direct interaction with complement components such as C1q, C8, and factor H [6]. Recently, we demonstrated that a specific subclass of human IgG, namely IgG2κ, copurifies with HRG^P as a HRG-IgG complex, and it is this complex that mediates the uptake of necrotic cells by phagocytes in a FcγRI-dependent manner [7]. In this study, the molecular mechanisms underpinning HRG^P-mediated necrotic cell removal have been characterized further, and our new data show that HRG functions cooperatively with cell surface HS on phagocytes to enhance the clearance of necrotic cells. In addition, HRG^P-aided recognition and uptake of necrotic cells can be inhibited by certain HRG ligands, such as heparin and hemin, an effect that may have functional consequences in vivo.

MATERIALS AND METHODS

Reagents

An N-terminal domain-specific anti-human HRG mAb (HRG-4) was provided by AGEN (Brisbane, Australia). An anti-HS mAb (F58-10E4) was purchased from Seikagaku (Tokyo, Japan). FITC-conjugated sheep $F(ab')_2$ anti-mouse Ig, PE-conjugated sheep $F(ab')_2$ anti-mouse Ig, and HRP-conjugated sheep anti-mouse Ig Abs were purchased from Millipore (Bedford, MD, USA). Heparin, biotinylated heparin, dermatan sulfate, chondroitin-4-sulfate, chondroitin-6-sulfate, hemin, ExtraAvidin®, BSA, chondroitinase ABC, and PE-conjugated rabbit anti-human IgG Ab were purchased from Sigma-Aldrich (St. Louis, MO, USA). Endotoxin-free heparin was provided by Katharine Goodall (Department of Biochemistry, La Trobe University, Australia). Platelet-derived human heparanase was a gift from Dr. Craig Freeman (John Curtin School of Medical Research, Canberra, Australia).

Cell lines

Jurkat T and THP-1 cell lines were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA). CHO-K1 and the GAG-deficient CHO cell lines (pgsA-745) were cultured in 50% DMEM and 50% Ham's nutrient mixture F-12 (Invitrogen). All culture media were supplemented with 10% FCS, 5 mM L-glutamine, 30 μg/ml penicillin G, 50 μg/ml streptomycin sulfate, and 50 μg/ml neomycin sulfate. Cell lines were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were induced to be necrotic by exposure to hyperthermic conditions (56°C, 30 min) in the absence of serum components. The necrotic nature of heat-killed cells was characterized by flow cytometry as per Poon et al. [7].

Purification of primary human monocytes

CD14 MicroBeads (Miltenyi Biotec, Auburn, CA, USA) were used for the positive selection of human monocytes from human PBMCs, according to the manufacturer's instructions.

Protein purification

HRG^P was purified from human plasma, according to a method described previously [8, 9]. Briefly, human plasma was passed through a phosphocellulose column (Whatman, Maidstone, UK), equilibrated with 0.5 M NaCl, 10 mM sodium phosphate buffer, 1 mM EDTA, pH 6.8. Bound HRG was eluted with 2.0 M NaCl, 10 mM sodium phosphate buffer, 1 mM EDTA, pH 6.8. An EndoTrap red column (Profos AG, Regensburg, Germany) was used to remove traces of endotoxin present in HRG^P, according to the manufacturer's instructions. Traces of human IgG were removed by passing HRG^P through a HiTrap™ Protein G column (Amersham Biosciences, Little Chalfont, UK) to yield HRG^{PID} preparations. The IgG^{HRG} was purified using the HiTrap™ Protein G column.

ELISA

ELISAs were performed according to a method described previously [10]. Briefly, U-bottomed 96-well polyvinyl chloride microtiter plates (Dynex Technologies, Denkendorf, Germany) were coated with ExtraAvidin® (10 μg/ml) for 90 min at 4°C prior to addition of PBS/3% (w/v) BSA for 3 h at 4°C to block nonspecific binding. Plates were washed with PBS/0.05% Tween-20 and then incubated with biotinylated heparin (10 μg/ml), diluted in PBS/1% BSA for 16 h at 4°C. HRG, diluted in PBS/1% BSA, was then added and the plates incubated for 90 min at 4°C. Plate-bound HRG was detected using the N-terminal domain-specific anti-human HRG mAb (HRG-4), followed by HRP-conjugated sheep anti-mouse Ig Ab. Plate-bound HRP was detected using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) peroxidase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA). The absorbance of the enzymatic product at 405 nm was measured using a Thermomax microplate reader (Molecular Devices, Sunnyvale, CA, USA). Data were analyzed by SoftMaxPro 4.0 software (Molecular Devices).

Immunofluorescence flow cytometry

Viable and necrotic cells were analyzed for protein binding by immunofluorescence flow cytometry as described previously [5]. Typically, 2×10^5 cells were incubated with HRG^P, HRG^{PID}, or IgG^{HRG}, diluted in PBS/0.1% BSA for 30 min at 4°C. Cells were washed three times with PBS/0.1% BSA and then detected for cell-bound HRG and IgG using an anti-human HRG mAb (HRG-4) or PE-conjugated rabbit anti-human IgG Ab, respectively. $F(ab')_2$ anti-human HRG mAb (HRG-4) was also used to detect HRG^{PID} binding to THP-1 cells. Cell surface expression of HS on THP-1, CHO-K1, and pgsA-745 cells was measured using an anti-HS mAb (F58-10E4). Cell-bound mAb was detected by PE- or FITC-conjugated sheep $F(ab')_2$ anti-mouse Ig Ab. Cells were resuspended in PBS/0.1% BSA containing 1 μg/ml Hoechst 33258 (Calbiochem, La Jolla, CA, USA) and analyzed immediately by flow cytometry using a LSRI flow cytometer and CellQuest Pro software (BD Biosciences, San Diego, CA, USA). The resultant flow cytometry data were analyzed by FlowJo software (Tree Star, Ashland, OR, USA). Cells were gated appropriately based on forward- and side-scatter. Live and dead cells were distinguished based on Hoechst 33258-negative and -positive staining, respectively.

Phagocytosis assay

Phagocytes (THP-1 cells and $CD14^+$ primary monocytes) were labeled with PKH26 (Sigma-Aldrich), according to the manufacturer's instructions and Jurkat T cells with CFSE (Molecular Probes, Eugene, OR, USA), as described previously [5, 7]. CFSE-labeled Jurkat T cells were induced into necrosis by exposure to hyperthermic conditions (56°C, 30 min). The phagocytosis assay was performed immediately under serum-free conditions by incubating PKH26-labeled phagocytes with CFSE-labeled necrotic Jurkat T cells at a cell ratio of 1:5–1:10. Samples were then incubated for 60 min at 37°C in a humidified atmosphere containing 5% CO_2. Samples were placed immediately on ice and analyzed by flow cytometry using a LSRI flow cytometer and CellQuest Pro software (BD Biosciences). The resultant flow cytometry data were analyzed by FlowJo software (Tree Star). Percentage phagocytosis was determined as the percentage of PKH26-positive phagocytic cells that had ingested CFSE-positive necrotic Jurkat T cells. In certain experiments, the GAG-deficient CHO cell line (pgsA-745) was used as the necrotic cells.

RESULTS

Heparin inhibits the ability of HRG^P to enhance the phagocytosis of necrotic cells

HRG is a multidomain protein that can bind to a wide variety of ligands, including haem, Zn^{2+}, phospholipids, plasminogen, fibrinogen, thrombospondin, IgG, and C1q [2, 7]. Importantly, HRG is the second-most abundant plasma protein that can bind heparin and HS with high affinity [11, 12]. Thus, the effect of HRG ligands, such as heparin, on the ability of HRG^P to enhance necrotic cell clearance was investigated. Under serum-free conditions, the presence of heparin (12.5 kDa) at the highest concentration of 20 μg/ml inhibited HRG^P-mediated phagocytosis of necrotic cells to below control levels (**Fig. 1A** and **B**), indicating that ligand binding can regulate the ability of HRG^P to enhance necrotic cell clearance. Interestingly, heparin (12.5 kDa) also significantly reduced necrotic cell uptake in the absence of HRG^P (Fig. 1A and B), 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. To examine the possible effect of contaminating LPS on the phagocytic assay, endotoxin-free HRG^P and endotoxin-free heparin (12.5 kDa) were used, and similar results were observed as described above (Supplemental Fig. 1). These data indicate that the effect of HRG^P and heparin on the phagocytosis of necrotic cells is not caused by the presence of trace amounts of LPS. To investigate further whether the effect of heparin is physiologically relevant, phagocytic assays were performed using primary human monocytes. Similar to the findings using THP-1 cells, the presence of heparin (12.5 kDa, 50 μg/ml) markedly inhibited the phagocytosis of necrotic cells by primary human monocytes in the presence or absence of HRG^P (Supplemental Fig. 2), suggesting that the effect of heparin on necrotic cell clearance is not limited to the monocytic cell line THP-1.

Heparin inhibits 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 has important implications, with heparin being routinely administrated clinically as an anticoagulant agent [13] or released by activated mast cells to regulate various biological processes [14, 15]. As necrotic cell uptake enhanced by HRG^P involves complex interactions among the necrotic cell, HRG, the copurified IgG present in the HRG^P preparation, and the phagocyte, the effect of heparin on these interactions was examined further. First, the effect of heparin on the binding of HRG and HRG-IgG complexes to necrotic Jurkat T cells and viable THP-1 cells was investigated. The presence of heparin (12.5 kDa) at a concentration of 50 μg/ml significantly reduced the binding of HRG and HRG-IgG complexes in HRG^P preparations (100 μg/ml) to necrotic cells by ~55% and 70%, respectively (**Fig. 2A–C**). 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). Alternatively, the HRG-binding site on necrotic cells may be 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. 2D), indicating that the inhibitory effect of heparin on the binding of HRG and HRG-IgG complexes to necrotic cells

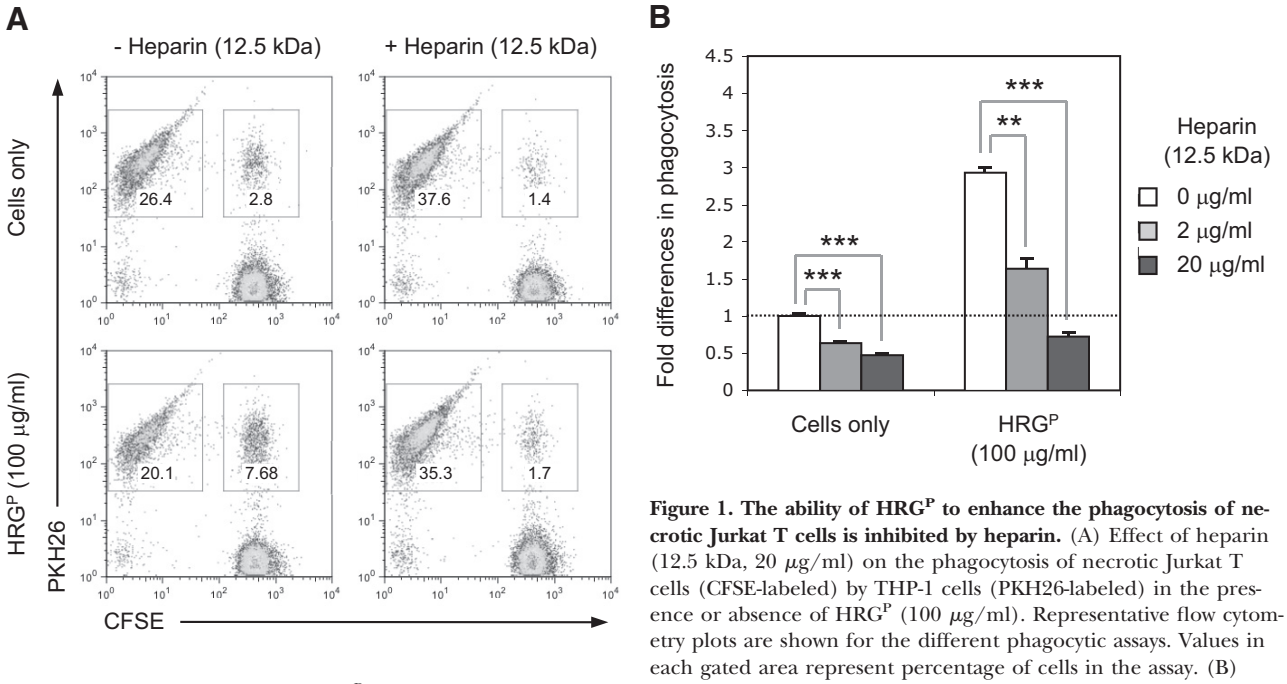

Figure 1. The ability of HRG^P to enhance the phagocytosis of necrotic Jurkat T cells is inhibited by heparin. (A) Effect of heparin (12.5 kDa, 20 μg/ml) on the phagocytosis of necrotic Jurkat T cells (CFSE-labeled) by THP-1 cells (PKH26-labeled) in the presence or absence of HRG^P (100 μg/ml). Representative flow cytometry plots are shown for the different phagocytic assays. Values in each gated area represent percentage of cells in the assay. (B) Quantitative comparison of the HRG^P-enhanced phagocytosis of necrotic Jurkat T cells in the presence of an increasing concentration of heparin (12.5 kDa), and data are expressed as fold-difference in the level of phagocytosis, relative to the necrotic cells-only control. Error bars represent SEM (n=3); $^{**}P < 0.01$; $^{***}P < 0.001$.

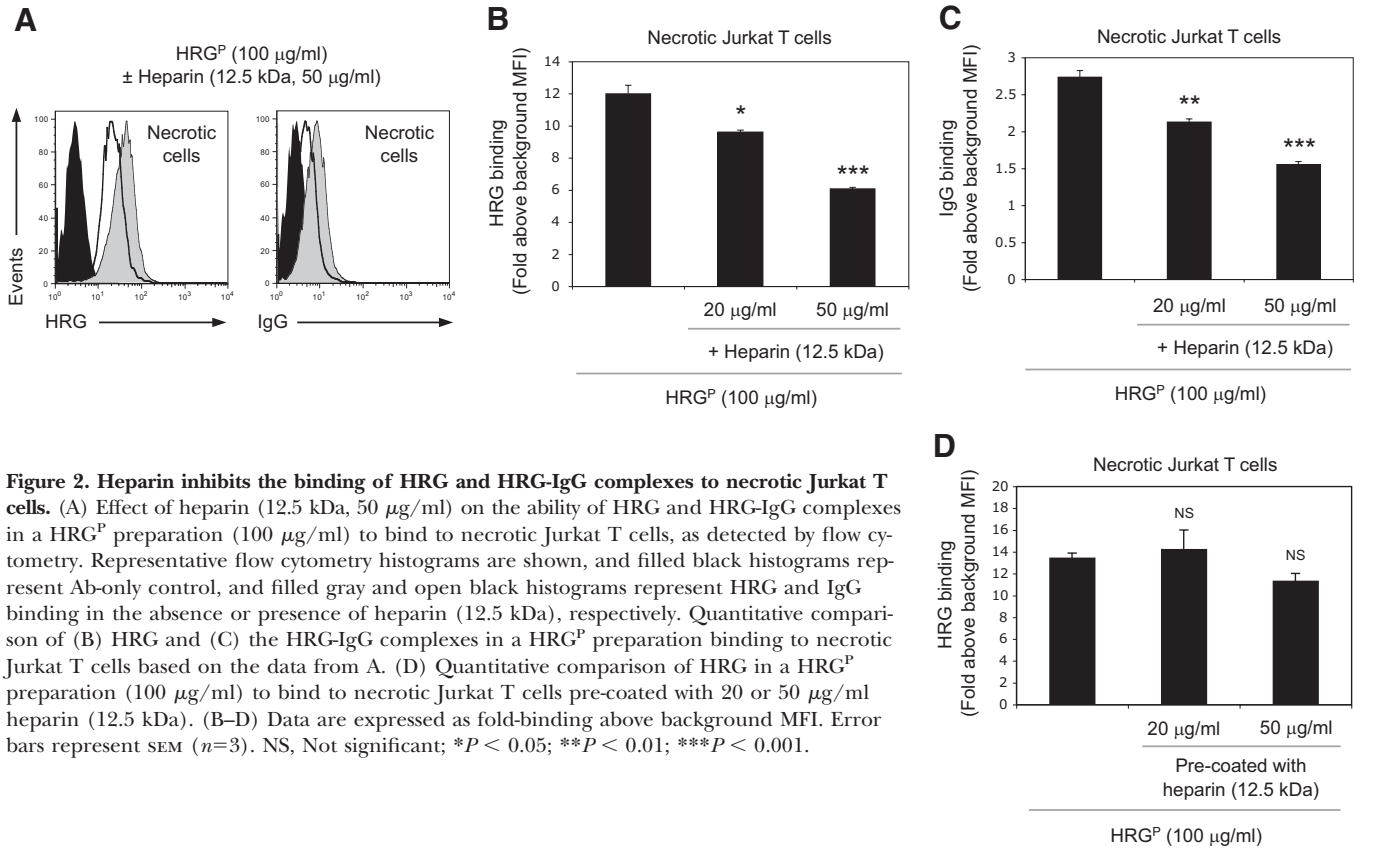

Figure 2. Heparin inhibits the binding of HRG and HRG-IgG complexes to necrotic Jurkat T cells. (A) Effect of heparin (12.5 kDa, 50 μg/ml) on the ability of HRG and HRG-IgG complexes in a HRG^P preparation (100 μg/ml) to bind to necrotic Jurkat T cells, as detected by flow cytometry. Representative flow cytometry histograms are shown, and filled black histograms represent Ab-only control, and filled gray and open black histograms represent HRG and IgG binding in the absence or presence of heparin (12.5 kDa), respectively. Quantitative comparison of (B) HRG and (C) the HRG-IgG complexes in a HRG^P preparation binding to necrotic Jurkat T cells based on the data from A. (D) Quantitative comparison of HRG in a HRG^P preparation (100 μg/ml) to bind to necrotic Jurkat T cells pre-coated with 20 or 50 μg/ml heparin (12.5 kDa). (B–D) Data are expressed as fold-binding above background MFI. Error bars represent SEM (n=3). NS, Not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

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^{PID} 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 Abs to FcγR on the THP-1 cells. The presence of heparin (12.5 kDa, 50 μg/ml) reduced the binding of HRG in a HRG^{PID} preparation (100 μg/ml) to viable THP-1 cells by >95% (**Fig. 3A** and **B**), suggesting that the binding of HRG to viable THP-1 cells is likely to be mediated via cell surface GAG. In contrast, the presence of heparin (12.5 kDa, 50 μg/ml) had only a small, albeit significant, inhibitory effect on the binding of HRG-IgG complexes in HRG^P preparations (100 μg/ml) and the IgG^{HRG} (2 μg/ml) to viable THP-1 cells (Fig. 3A, C, and D). Collectively, these data show that the ability of heparin to inhibit necrotic cell uptake aided by HRG^P may involve a number of different molecular mechanisms.

Cell surface HS on phagocytes functions cooperatively with HRG to aid necrotic cell clearance

Heparin, apart from being a well-characterized ligand of HRG [2], can, itself, bind strongly to permeabilized cells, possibly to nuclear-derived materials, and the HS component on the surface of macrophages has been proposed to facilitate the phagocytosis of dying cells [16]. 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°C following heparanase treatment were investigated. As indicated in **Fig. 4A**, 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 min at 37°C following heparanase treatment. As expected, HRG^{PID} (100 μg/ml) binding to THP-1 cells was reduced markedly by pretreating THP-1 cells with human heparanase, the reduction in binding comparable with that achieved by heparin inhibition (Fig. 4B), 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 μg/ml), whereas untreated and heparanase-treated THP-1 cells exhibited a similar level of necrotic cell uptake in the absence of HRG^P (Fig. 4C and D), indicating that cell surface HS on THP-1 cells and HRG^P may act in concert to aid the disposal of necrotic cells. Although these results suggest that cell surface HS on phagocytes alone may not be sufficient to trigger phagocytosis of necrotic cells, it should be noted that the data presented in Fig. 4 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 HRG^P-dependent or -independent mechanisms.

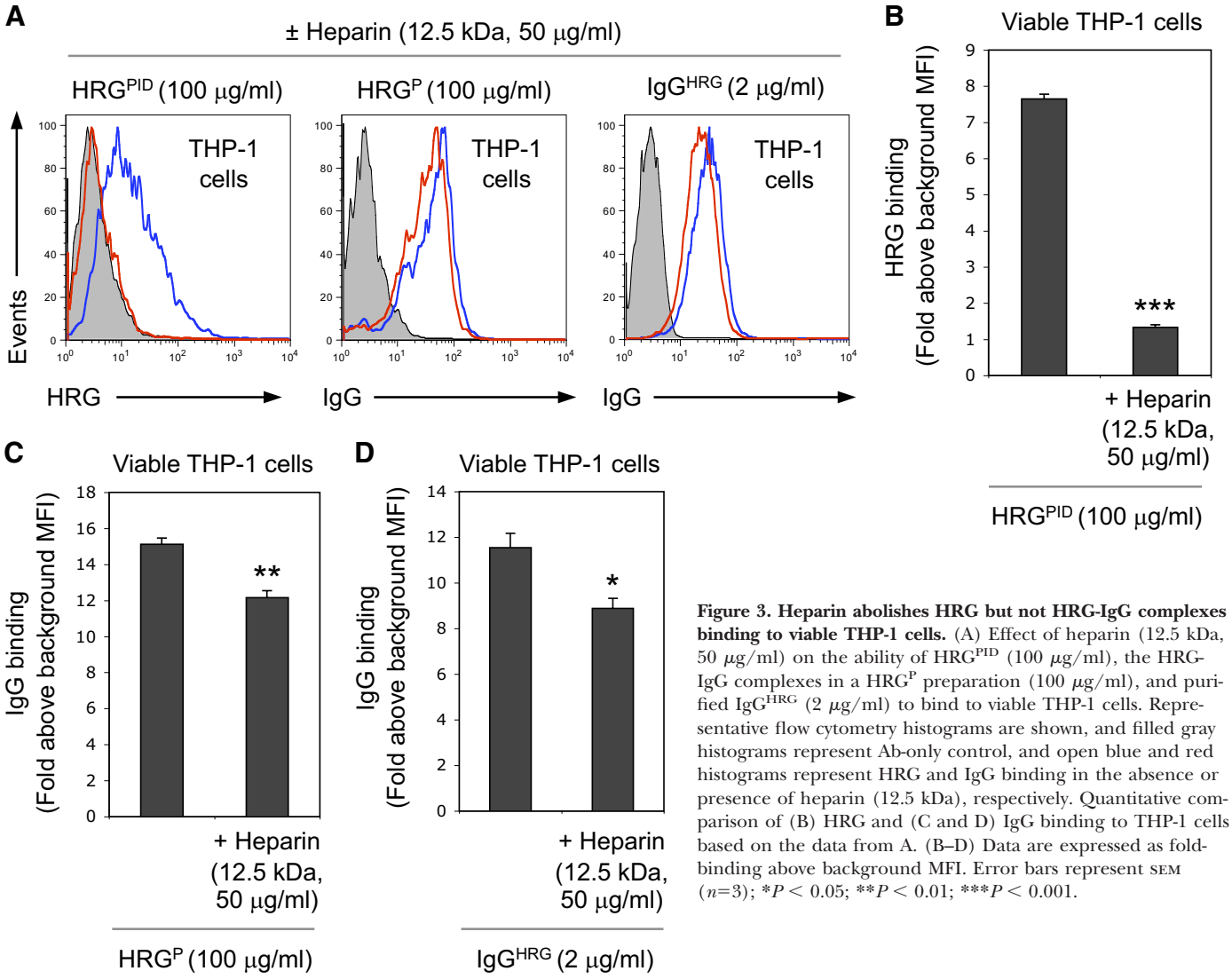

Figure 3. Heparin abolishes HRG but not HRG-IgG complexes binding to viable THP-1 cells. (A) Effect of heparin (12.5 kDa, 50 μg/ml) on the ability of HRG^{PID} (100 μg/ml), the HRG-IgG complexes in a HRG^{P} preparation (100 μg/ml), and purified IgG^{HRG} (2 μg/ml) to bind to viable THP-1 cells. Representative flow cytometry histograms are shown, and filled gray histograms represent Ab-only control, and open blue and red histograms represent HRG and IgG binding in the absence or presence of heparin (12.5 kDa), respectively. Quantitative comparison of (B) HRG and (C and D) IgG binding to THP-1 cells based on the data from A. (B–D) Data are expressed as fold-binding above background MFI. Error bars represent SEM (n=3); $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

To examine further the role of cell surface HS on THP-1 cells in mediating necrotic cell uptake via HRG^{P}, phagocytic assays were performed in the presence of soluble HS. Similar to heparin, HS (20 μg/ml) blocked the ability of HRG^{P} (100 μg/ml) to enhance the phagocytosis of necrotic cells (**Fig. 5**). In support of a role for cell surface HS in this process, the presence of dermatan sulfate, chondroitin-4-sulfate, and chondroitin-6-sulfate (20 μg/ml) or pretreating THP-1 cells with chondroitinase ABC had no apparent effect on HRG^{P}-aided phagocytosis of necrotic cells (Fig. 5). These data further suggest that HRG^{P} functions cooperatively with HS but not dermatan sulfate or chondroitin sulfate on THP-1 cells to aid necrotic cell clearance.

Necrotic cell HS is not involved in HRG^{P}-mediated necrotic cell removal

In addition to binding to HS on phagocytic cells, HRG can bind to cell surface HS on necrotic cells, and this interaction may play an important role in the ability of HRG^{P} to enhance the disposal of necrotic cells. Thus, 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^{P} preparations (100 μg/ml) bound to untreated and heparanase-treated necrotic Jurkat T cells to a similar extent (**Fig. 6A**), suggesting that unlike viable cells (see Fig. 4), HS is not the key HRG ligand present on necrotic cells. Surprisingly, heparanase treatment actually enhanced the binding of HRG-IgG complexes in HRG^{P} preparations (100 μg/ml) to necrotic Jurkat T cells (Fig. 6B). 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^{P} preparations (100 μg/ml) to untreated and heparanase-treated necrotic Jurkat T cells (Fig. 6A and B), suggesting that heparin can block the HRG-binding site for necrotic cells, although the necrotic cell ligand is not HS. In phagocytic assays, heparanase treatment of necrotic Jurkat T cells had no apparent effect on control or HRG^{P}-enhanced uptake of necrotic

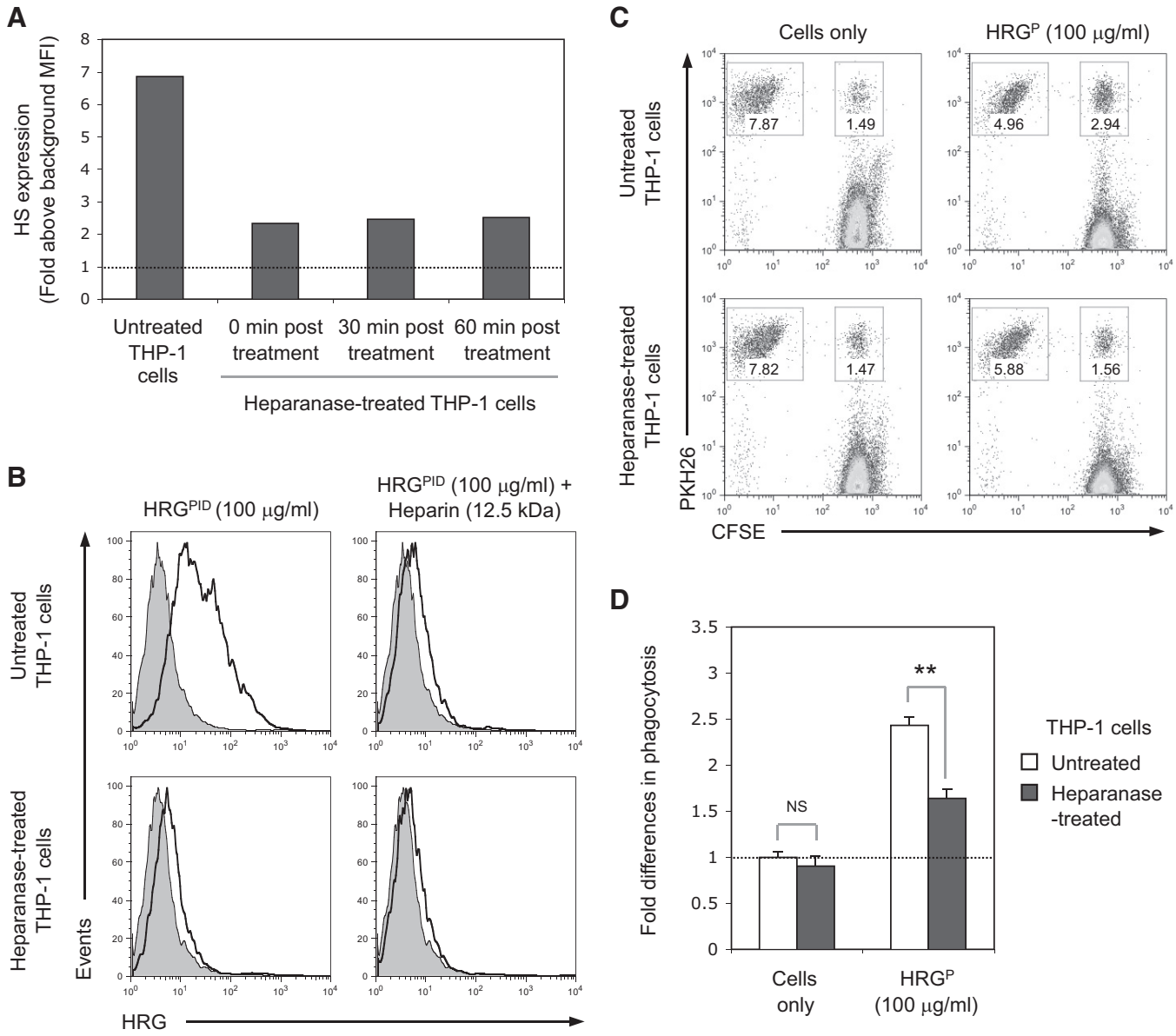

Figure 4. Cell surface HS on phagocytic THP-1 cells plays an important role in HRG^P-mediated phagocytosis of necrotic Jurkat T cells. (A) Analysis of cell surface expression of HS on untreated THP-1 cells or THP-1 cells incubated at 37°C for 0, 30, or 60 min following human platelet-derived heparanase treatment (4 μg/ml, 37°C, 60 min), and the level of HS expression was detected by flow cytometry. Data are expressed as fold-binding above background MFI. (B) Effect of heparanase pretreatment (4 μg/ml) at 37°C for 60 min and heparin (12.5 kDa, 50 μg/ml) on the ability of HRG^{PID} (100 μg/ml) to bind to viable THP-1 cells. Representative flow cytometry histograms are shown, and filled gray histograms represent Ab-only control and open black histograms, HRG binding. (C) Ability of heparanase-treated THP-1 cells (PKH26-labeled) to uptake necrotic Jurkat T cells (CFSE-labeled) in the presence or absence of HRG^P (100 μg/ml). Representative flow cytometry plots 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 Jurkat T cells, based on the data from C, and data are expressed as fold-difference in the level of phagocytosis relative to the necrotic cells-only control and the assay performed using untreated THP-1 cells. Error bars represent SEM (n=3); $^{**}P < 0.01$.

cells (Fig. 6C), indicating that necrotic cell clearance under control conditions or via HRG^P is independent of HS expressed on the necrotic cells.

To verify further that cell surface HS on necrotic cells plays little or no role in HRG^P-mediated necrotic cell uptake, the GAG-deficient CHO cell line (pgsA-745), which expresses no detectable cell surface HS compared with the wild-type HS-bearing CHO-K1 cells (Fig. 6D), was used as the necrotic target cells. HRG and HRG-IgG complexes in HRG^P preparations (100 μg/ml) bound strongly to necrotic pgsA-745 cells, and this binding was inhibited significantly by heparin (12.5 kDa, 50 μg/ml; Fig. 6E 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

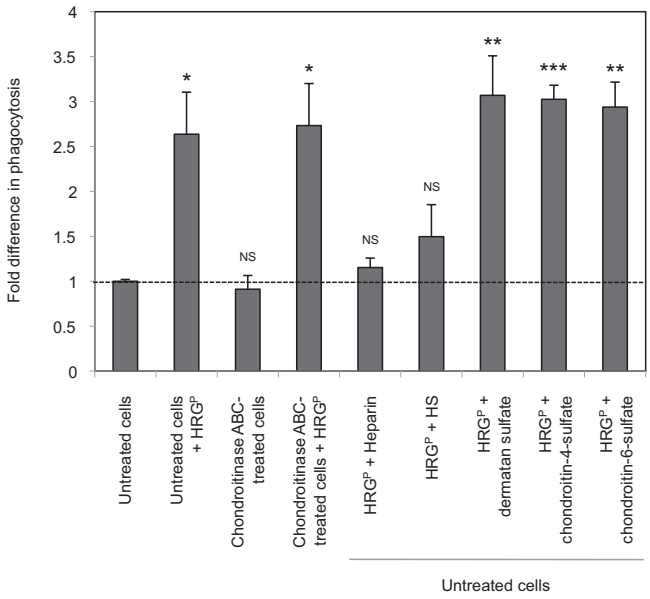

Figure 5. Heparan sulfate but not chondroitin sulfate or dermatan sulfate is involved in the enhanced uptake of necrotic cells induced by HRG^P. The figure depicts the effect of 20 μg/ml heparin (12.5 kDa), HS, dermatan sulfate, chondroitin-4-sulfate, and chondroitin-6-sulfate or pretreating THP-1 cells with chondroitinase ABC (2 unit/ml, 37°C, 60 min) on the ability of HRG^P (100 μg/ml) to enhance necrotic cell uptake. Data are expressed as fold-difference in the level of phagocytosis relative to the necrotic cells-only control and the assay performed using untreated THP-1 cells. Error bars represent SEM (n=3); *$P <$ 0.05; **$P < 0.01$; ***$P < 0.001$.

mediated by HRG^P was abolished completely by heparin (12.5 kDa, 20 μg/ml; Fig. 6G). 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 on necrotic cell uptake is unlikely to be mediated through the blocking of HRG binding to cell surface HS on necrotic cells.

Hemin inhibits HRG binding to necrotic cells and heparin

To assess whether other ligands of HRG could also influence HRG^P-mediated enhancement of necrotic cells, hemin (the Fe^{3+} oxidation product of haem), was also tested in the HRG-binding and necrotic cell uptake assays. As described for heparin, hemin also significantly reduced necrotic cell removal mediated by HRG^P (100 μg/ml) but had no effect on control necrotic cell uptake (**Fig. 7A**). These data further support the notion that the direct binding of certain ligands to HRG could regulate its ability to enhance phagocytosis of necrotic cells. Similar to the effect of heparin as described above, hemin (10 μg/ml) partially inhibited the binding of HRG and HRG-IgG complexes in HRG^P preparations (100 μg/ml) to necrotic Jurkat T cells (Fig. 7B and C). In additional experiments, hemin was found to inhibit HRG^{PID} binding totally to immobilized heparin (Fig. 7D). Collectively, these results indicate that the interaction between HRG and hemin may modify the ligand-binding capacity of HRG via a conformational change and/or directly mask the necrotic cell/heparin-binding sites on HRG. Furthermore, these data also suggest that hemin can potentially modify the ability of HRG to aid necrotic cell removal via a similar mechanism as heparin.

HRG^P-aided necrotic cell uptake is potentiated moderately by Zn^{2+}

It has been proposed that HRG can function as a Zn^{2+} sensor in response to tissue injury [2], and the ability of HRG to regulate angiogensis and microbial killing can be enhanced by Zn^{2+} [17–20]. Moreover, the presence of a physiological concentration of Zn^{2+} (20 μM) has been shown previously to potentiate HRG binding to cell surface HS [8]. Thus, the effect of Zn^{2+} on HRG^P-enhanced necrotic cell clearance was investigated. In contrast to the other HRG ligands described above, the presence of 20 μM Zn^{2+} enhanced HRG^P-aided phagocytosis of necrotic cells only slightly (100 μg/ml) but had no apparent effect on control necrotic cell uptake (**Fig. 8**). These data suggest that the presence of Zn^{2+}, possibly at sites of tissue damage, may promote necrotic cell disposal moderately by HRG.

DISCUSSION

Numerous pattern recognition molecules, such as TLRs, C-reactive protein, and C1q, have been shown to play an important role in detecting and eliminating foreign pathogens, as well as dying host cells [1]. However, the molecular components that are involved in sensing and clearing necrotic cells are often poorly characterized. In this study, we have demonstrated that in addition to FcγR, HRG functions in concert with cell surface HS on phagocytes to enhance necrotic cell disposal. Furthermore, the presence of HRG ligands, such as heparin and hemin, can regulate this process by interfering with certain interactions (summarized in **Fig. 9**).

As HRG can interact with numerous molecules [2], it is not surprising that the binding of certain ligands to HRG could compete for the ability of HRG to bind other ligands and interfere with the adaptor function of HRG. In addition to blocking HRG binding to cell surface HS on phagocytes or to ligand(s) exposed on necrotic cells, heparin and hemin have been shown previously to inhibit the binding of IgG^{HRG} to HRG^{PID}, as measured by ELISA [7]. Thus, it is worth noting that the reduction in HRG-IgG complexes binding to necrotic cells and the inhibition of HRG^P-mediated necrotic cell uptake by heparin/hemin could also be the result of heparin/hemin disrupting the formation of HRG-IgG complexes. Furthermore, the ability of heparin to block the interaction between HRG and necrotic cells suggests that in addition to the N1N2 domain of HRG [5], the HRR of HRG may be involved in necrotic cell binding, as the HRR represents a major heparin/HS-binding site on HRG [20–22]. Although previous studies by Jones et al. [8] have demonstrated that the N1N2 domain of HRG can also bind heparin avidly [8], the inability of heparin to reduce N1N2 domain-binding to necrotic cells [5] sug-

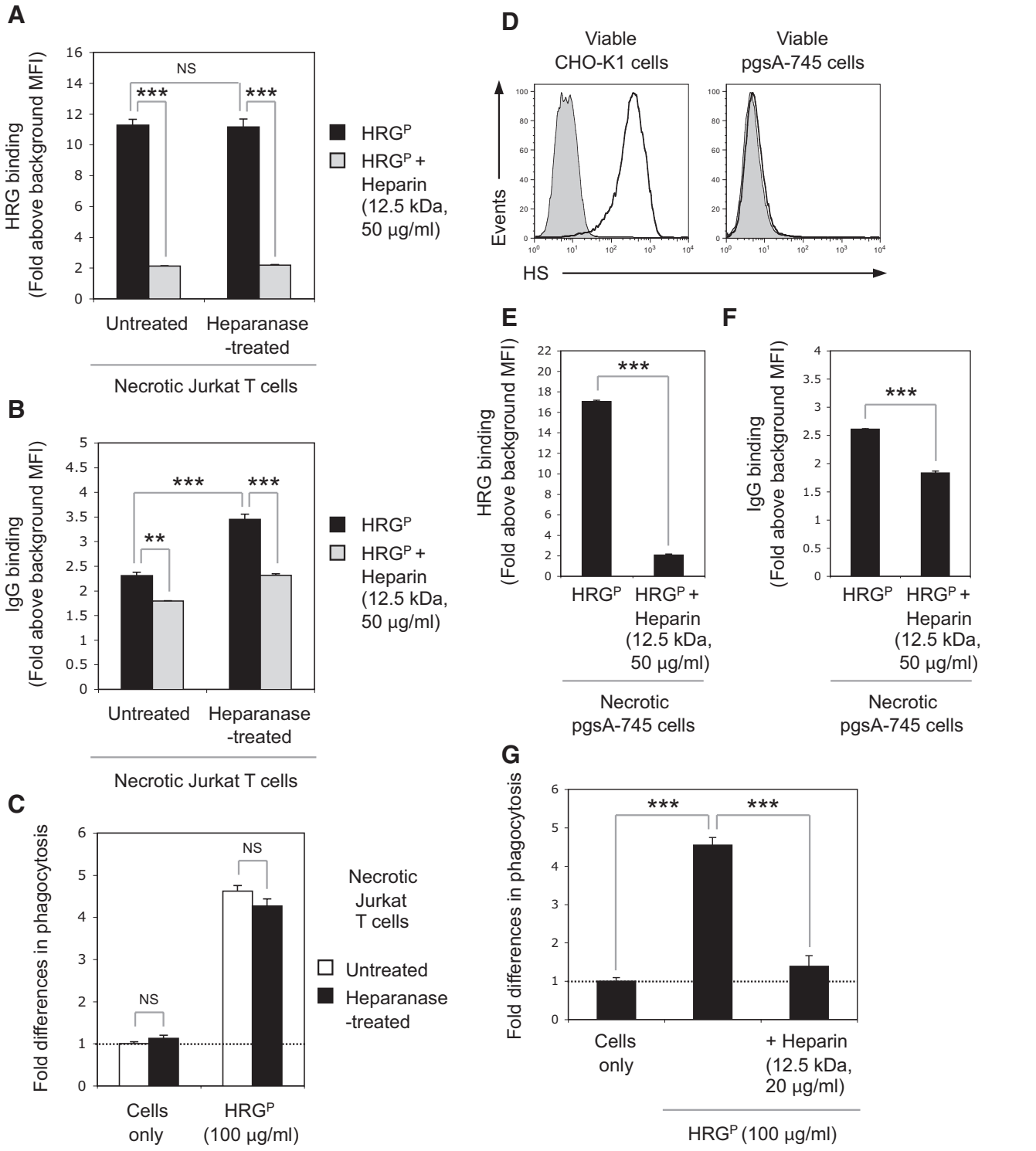

Figure 6. Necrotic cell HS is not involved in the enhanced phagocytosis of necrotic cells induced by HRG^P. Effect of heparanase pretreatment (4 μg/ml, 37°C, 60 min) and heparin (12.5 kDa, 50 μg/ml) on the ability of (A) HRG and (B) the HRG-IgG complexes in a HRG^P preparation (100 μg/ml) to bind to 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^P (100 μg/ml). (D) Cell surface expression of HS by viable CHO-K1 cells and a GAG-deficient CHO cell line (pgsA-745), and the level of HS expression is detected by flow cytometry. Representative flow cytometry histograms are shown, and filled histograms represent secondary Ab-only control and open black histograms, HS expression. Effect of heparin (12.5 kDa, 50 μg/ml) on the ability of (E) HRG and (F) the HRG-IgG complexes in a HRG^P preparation (100 μg/ml) to bind to necrotic pgsA-745 cells. (G) Effect of heparin (12.5 kDa, 50 μg/ml) on HRG^P (100 μg/ml)-mediated phagocytosis of necrotic pgsA-745 cells. For flow cytometry-based cell-binding assays in A, B, E, and F, data are expressed as fold-binding above background MFI. Error bars represent SEM (n=3). (C and G) For phagocytic assays, data are expressed as fold-difference in the level of phagocytosis relative to the untreated necrotic Jurkat T cells or necrotic pgsA-745 cells-only control, and error bars represent SEM (n=3); $^{**}P < 0.01$; $^{***}P < 0.001$.

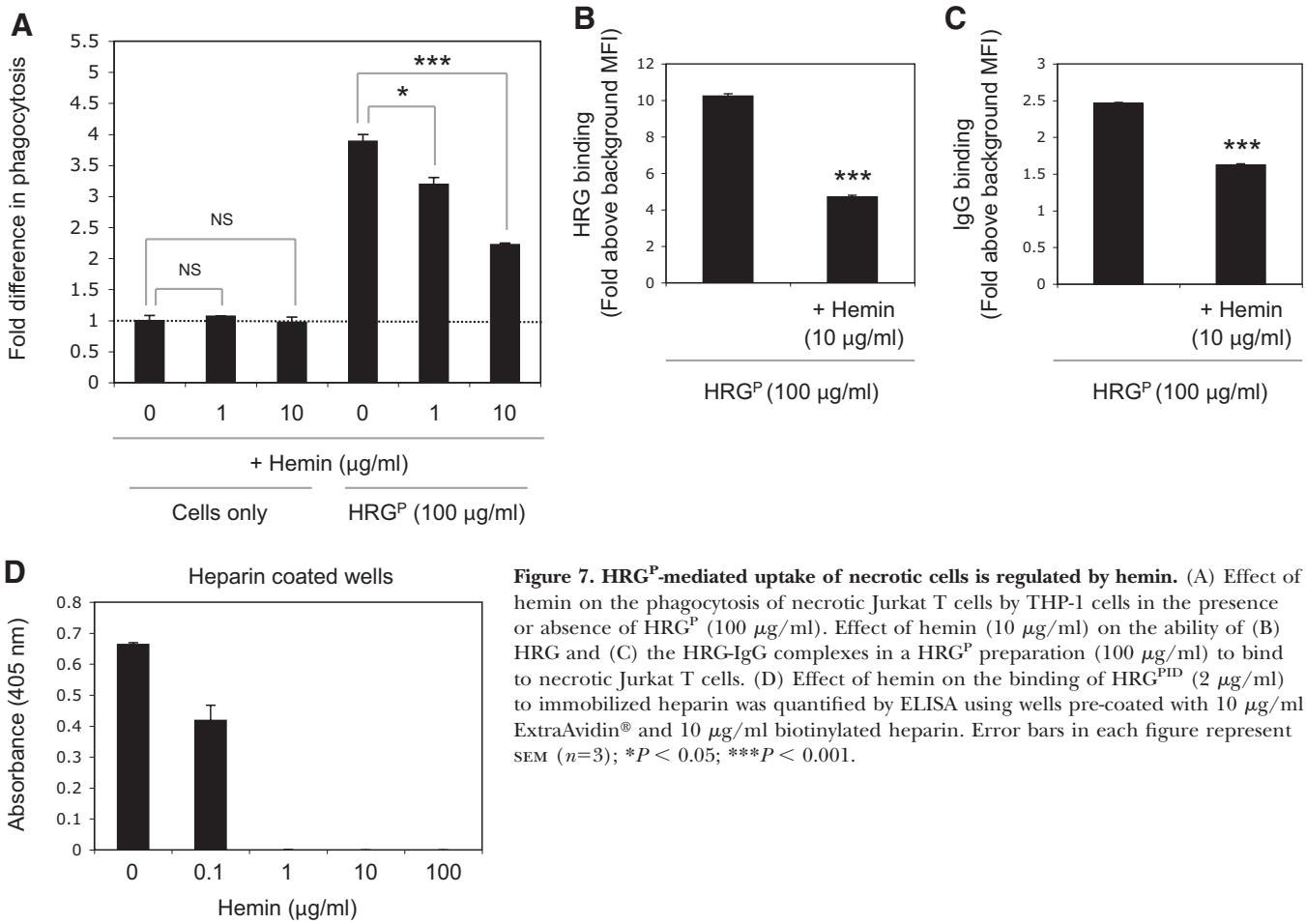

Figure 7. HRG^P-mediated uptake of necrotic cells is regulated by hemin. (A) Effect of hemin on the phagocytosis of necrotic Jurkat T cells by THP-1 cells in the presence or absence of HRG^P (100 μg/ml). Effect of hemin (10 μg/ml) on the ability of (B) HRG and (C) the HRG-IgG complexes in a HRG^P preparation (100 μg/ml) to bind to necrotic Jurkat T cells. (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 ExtraAvidin® and 10 μg/ml biotinylated heparin. Error bars in each figure represent SEM (n=3); *$P < 0.05$; ***$P < 0.001$.

gests that the inhibitory effect of heparin on the binding of HRG to necrotic cells (Figs. 2 and 6) may be a result of blocking a necrotic cell-binding site(s) located within the HRR on HRG. Similarly, hemin is also thought to bind to the HRR of HRG [23], and based on its small size, it is unlikely to have any significant steric effects on the ligand-binding properties of HRG. Thus, the ability of hemin to reduce HRG binding to necrotic cells (Fig. 7) indicates that the HRR itself could be important for necrotic cell recognition. Alternatively, the binding of heparin/hemin to the HRR of HRG may cause a ligand-induced conformational change and regulates its ability to bind to necrotic cells. In our previous studies [7], certain intracellular phospholipids, such as phosphatidyl inositol 4-phosphate, phosphatidic acid, and cardiolipin, have been proposed as potential ligands of HRG exposed on necrotic cells. It would therefore be of interest to examine whether heparin/hemin can regulate HRG binding to these intracellular phospholipids and identify the region(s) on HRG that may mediate HRG-lipid interactions.

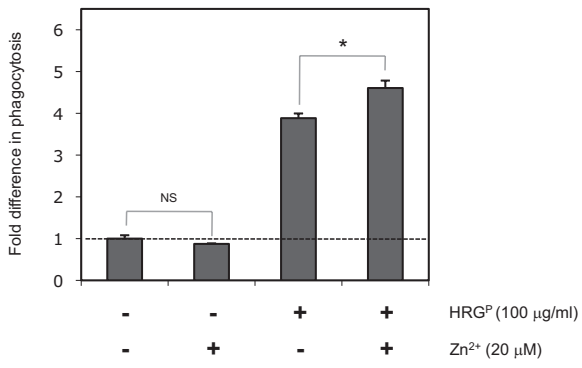

Figure 8. The presence of Zn^{2+} moderately enhances HRG^P-mediated necrotic cell uptake. The figure depicts the effect of Zn^{2+} (20 μM) on the ability of HRG^P (100 μg/ml) to enhance necrotic cell uptake. Data are expressed as fold-difference in the level of phagocytosis relative to the necrotic cells-only control. Error bars represent SEM (n=3); *$P < 0.05$.

Consistent with previous studies by Jones et al. [5], the findings presented herein suggest that HS is not the major ligand recognized by HRG on necrotic cells, and the inhibitory effect of heparin is not simply blocking HRG binding to HS exposed on necrotic cells. In contrast, the marked reduction in HRG binding to THP-1 cells in the presence of heparin or following heparanase treatment of THP-1 cells strongly suggests that HRG can bind directly to cell surface HS on phagocytes. These results are consistent with our previous studies demon-

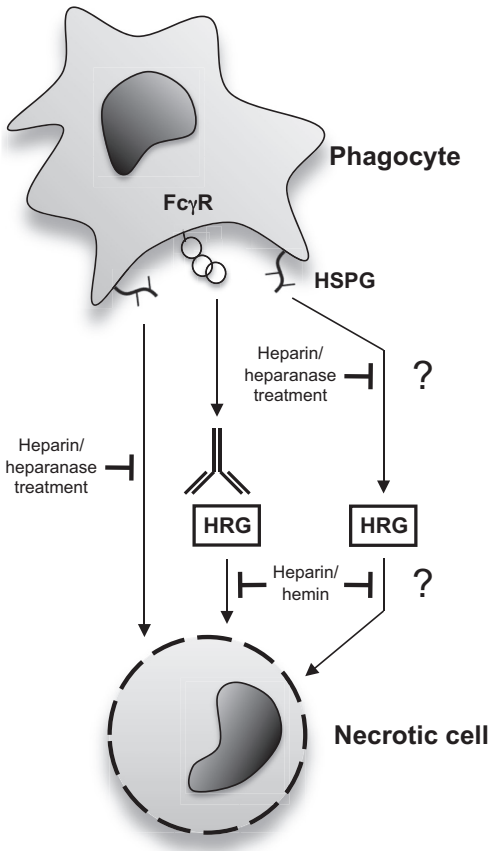

Figure 9. Proposed molecular mechanism of HRG^P-aided removal of necrotic cells. HRG^P functions in concert with cell surface FcγR and HS to enhance necrotic cell removal, processes that are inhibited by heparanase treatment (removal of cell surface HS) and blocked by heparin or hemin. HRG and HRG-IgG complexes in a HRG^P preparation aid the removal of necrotic cells via a HS- and FcγR-dependent mechanism, respectively. The direct binding of cell surface HSPG on phagocytes to necrotic cells functions cooperatively with HRG^P-mediated pathways to aid necrotic cell clearance. The presence of heparin blocks the ability of HSPG on phagocytes from interacting with necrotic cells. The removal of cell surface HS on phagocytes by heparanase treatment also reduces necrotic cell uptake enhanced by HRG^P. Heparin and hemin partially inhibit HRG and HRG-IgG complexes binding to necrotic cells.

strating that HS, but not FcγR, is the key HRG receptor on THP-1 cells [7]. Importantly, the removal of HS from the surface of THP-1 cells resulted in a significant decrease in phagocytosis of necrotic cells enhanced by HRG^P but had no obvious effect on necrotic cell uptake in the absence of HRG^P. Although these data suggest that HRG may act as an adaptor molecule between necrotic cells and cell surface HS on phagocytes, HRG^{PID} alone only enhanced the phagocytosis of necrotic cells by <1.5-fold, and an anti-FcγRI-blocking mAb was able to abolish HRG^P-mediated necrotic cell uptake completely [7]. Thus, it is more likely that HRG and HRG-IgG complexes and cell surface HS on phagocytes work cooperatively to bind necrotic cells through multiple interactions to facilitate their removal. Although previous studies by Gebska et al. [16] 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 important for necrotic cell clearance and involves serum opsonins such as HRG. As HS is present on most cell surfaces, it represents a potentially important receptor on professional and nonprofessional phagocytes in assisting the uptake of necrotic cells in conjunction with other phagocytic pathways. Indeed, we have shown that heparanase-treatment of phagocytes to remove cell surface HS also reduces human serum-mediated removal of necrotic cells (Supplemental Fig. 3). In addition, the current study highlights a potential role for mammalian heparanase in regulating necrotic cell clearance when the local level of heparanase is high, such as at sites of tissue remodeling, wound healing, tumor growth, and inflammation [24, 25]. Besides dying host cells, ligation of cell surface HS has also been shown to facilitate the uptake of latex beads by epithelial cells [26, 27], further supporting the idea that cell surface HS is involved in phagocytosis.

The experimental results in this study show that the presence of certain physiological ligands of HRG can directly regulate the ability of HRG to sense necrotic cells. However, the effect of Zn^{2+}, haem, and heparin/HS on necrotic cell clearance, enhanced by HRG in vivo following events, such as platelets degranulation [28], hemolysis [29, 30], anticoagulation treatment [13], mast cell degranulation [14, 15], and the release of HS fragments from the extracellular matrix by heparanase at sites of tissue remodeling [31, 32], remains to be determined. Furthermore, as a number of well-characterized opsonins of apoptotic and necrotic cells, such β_2-glycoprotein I, thrombospondin, C1q, and serum amyloid protein, can also bind heparin [33–36], the presence of heparin could potentially regulate other phagocytic pathways. In support of this notion, we have shown that the presence of heparin can also inhibit the ability of human serum to enhance the uptake of necrotic cells by phagocytes (Supplemental Fig. 3).

In summary, this study provides evidence that HRG^P-aided necrotic cell removal requires the cooperative function of a number of cell surface receptors on phagocytes and is regulated by various HRG ligands. Further definition of the molecular mechanisms underpinning necrotic cell removal mediated by HRG may have important implications for the development of autoimmune disease caused by defective clearance of dying cells.

AUTHORSHIP

I.K.H.P. designed and performed research, analyzed and interpreted data, and wrote the manuscript. C.R.P. and M.D.H. designed research, interpreted data, and wrote the manuscript.

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KEY WORDS:
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