The effects of Castanospermine, an oligosaccharide processing inhibitor, on mononuclear/endothelial cell binding and the expression of cell adhesion molecules

Adrian D. Hibberd^{a,b,*}, Paul R. Trevillian^{a,b}, David A. Clark^a, Patrick Mcelduff^c, William B. Cowden^d

^a Newcastle Transplant Unit, Division of Surgery, John Hunter Hospital, Lookout Road, New Lambton, NSW 2305, Australia

^b Disciplines of Medicine and Surgery, School of Medicine and Population Health, University of Newcastle, Callaghan, Newcastle NSW 2308, Australia

^c Clinical Research Design IT and Statistical Support, Clinical Trials Unit, Hunter Medical Research Institute, School of Medicine and Population Health, University of Newcastle, Callaghan, Newcastle NSW 2308, Australia

^d John Curtin School of Medical Research, Australian National University, Canberra, Australian Capital Territory, Australia

ARTICLE INFO

Article history:

Received 13 December 2011

Received in revised form 9 May 2012

Accepted 15 May 2012

Keywords:

Castanospermine

Mononuclear/endothelial cell binding

Cell adhesion molecules

ABSTRACT

Introduction: In this study we aimed to determine whether Castanospermine, a transplant immunosuppressive agent, impaired mononuclear/endothelial cell binding and expression of their cell adhesion molecules.

Methods: The binding of human umbilical vein endothelial cells with peripheral blood mononuclear cells was measured by a binding assay using Chromium 51 label; the membrane expression of cell adhesion molecules was measured by flow cytometry expressed as mean fluorescence intensity ratios.

Results: Castanospermine decreased mononuclear/endothelial cell binding if and only if both cell types were treated with Castanospermine: this impairment occurred if endothelial cells were treated with a range of doses of Castanospermine and mononuclear cells were treated with a constant dose of Castanospermine ($p < 0.001$ versus untreated $p = 0.978$) or vice versa ($p = 0.004$ versus untreated $p = 0.582$). Upon human umbilical vein endothelial cells Castanospermine reduced the mean fluorescence intensity ratios of E-selectin ($p = 0.003$), ICAM-1 ($p < 0.001$), ICAM-2 ($p = 0.004$) and PECAM-1 ($p < 0.001$) but increased it for P-selectin ($p < 0.001$). Upon peripheral blood mononuclear cells Castanospermine reduced the mean fluorescence intensity ratios of L-selectin ($P < 0.001$), LFA-1 α ($p < 0.001$), VLA-4 ($p < 0.001$), Mac-1 ($P < 0.001$) and CR4 ($p < 0.001$) but increased the mean fluorescence intensity ratios of PSGL-1 ($p < 0.001$) and PECAM-1 ($p = 0.001$). Similar changes in mean fluorescence intensity ratios were found in the subset of lymphocytes and monocytes but the reductions in LFA-1 α and VLA-4 on lymphocytes and Mac-1 and CR4 on monocytes were greater.

Conclusions: The reduction in mononuclear/endothelial cell binding mediated by CAST and the reduction in expression of multiple cell adhesion molecules on these cell types help to explain the mechanism of its established immunosuppressive effect.

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Abbreviations: CAM, cell adhesion molecules; CAST, Castanospermine; ER, endoplasmic reticulum; FACS, fluorescence activated cell sorter; FBS, foetal bovine serum; FCS, foetal calf serum; FITC, fluorescein isothiocyanate conjugated; HBSS, Hank's buffered salt solution; HS-GAG, heparan sulphate-glycosaminoglycan; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular adhesion molecule-1; ICAM-2, intercellular adhesion molecule-2; LFA-1 α , lymphocyte function associated antigen-1 α ; MAb, monoclonal antibody; MFI, mean fluorescence intensity ratio; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PECAM-1, platelet-endothelial cell adhesion molecule-1; PSGL-1, P selectin glycoprotein ligand-1; RPE, R-phycoerythrin; TNF α , tumour necrosis factor alpha; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late antigen-4.

* Corresponding author at: Newcastle Transplant Unit, John Hunter Hospital, Hunter New England Local Health Network, PO Box 1083, Newcastle, NSW 2300, Australia. Tel.: +61 412 896432; fax: +61 2 49297361.

E-mail addresses: Adrian.Hibberd@hnehealth.nsw.gov.au (A.D. Hibberd), Paul.Trevillian@hnehealth.nsw.gov.au (P.R. Trevillian), David.Clark@hnehealth.nsw.gov.au (D.A. Clark), Patrick.Mcelduff@newcastle.edu.au (P. Mcelduff), WB.Cowden@anu.edu.au (W.B. Cowden).

1. Introduction

Successful transplantation requires the use of immunosuppression in the vast majority of recipients. There is a continuing need to improve the immunosuppressive agents used because of their toxicity which includes cancer [1], nephrotoxicity [2], diabetes mellitus [3] and infection [4]. Castanospermine (CAST), a glycosidase inhibitor, is a novel immunosuppressive agent that prolongs cardiac allograft survival [5], acts synergistically with tacrolimus [6], and alleviates experimental autoimmune encephalomyelitis (EAE) [7] and adjuvant induced polyarthritis [8]. It is relatively non-toxic in the rat [5]. But the mechanism of its immunosuppressive effect is poorly understood.

The net effect of glycosidase inhibitors is to change the structure of N linked glycoproteins required for cell–cell interaction, enzyme production and cell turnover [9]. N linked glycoproteins are formed by the binding of oligosaccharides to polypeptides on polysomes within the endoplasmic reticulum (ER). The oligosaccharide, bound to the

lipid carrier dolichyl-P, is then processed by cleaving glucose in the ER (glucosidase 1, glucosidase 2), cleaving mannose in ER (mannosidase 1), glycosylation (N acetyl transferase) in the ER, cleaving mannose in Golgi apparatus (mannosidase 2) and further glycosylation (N acetyl transferase) in the Golgi [9]. Castanospermine, an indolizidine alkaloid from the Australian Moreton Bay chestnut tree is a competitive inhibitor of glucosidase 1 and glucosidase 2 in the ER [9]. It therefore inhibits the first step in this process leading to high mannose containing oligosaccharides and impairment in transport through the ER and Golgi: the resultant N linked glycoproteins are dysfunctional.

The vascular endothelial cell plays a critical role in rejection: alloreactive antibody activates it by binding to its surface initiating antibody mediated rejection; alloreactive cells bind to its surface by cell adhesion molecules (CAM) before entering the subendothelial matrix of the transplant; it can act as an antigen presenting cell; and, it can contribute to vessel vasoconstriction and allograft ischaemia [10,11]. There is evidence that CAST may act upon the endothelial cell; it impairs the ability of endothelial cells to degrade the extracellular matrix when studied in vitro [12] and, curiously, histopathological studies of cardiac allografts in CAST treated rats show clusters of lymphoid cells about venules and capillaries with reduction of the interstitial infiltrate [5]. Similar perivascular cell clusters are observed in CAST treated rats with EAE [7] and adjuvant induced polyarthritis [8]. These findings suggest that CAST may impair endothelial cell function or possibly interfere with intragraft cell migration.

A sequence of cell adhesion molecules (CAMs) is necessary for the binding and transit of leucocytes through the vascular endothelium into the allograft [13]. Initially leucocytes are tethered to the endothelium by the selectin family (P-, E-, and L-selectin); cytokines and chemokines from leucocytes and endothelial cells activate the expression of additional CAMs on both; firm adhesion between the two is effected by integrins upon leucocytes [lymphocyte function associated antigen-1 (LFA-1), very late antigen-4 (VLA-4)] and the immunoglobulin supergene family on endothelial cells [intercellular adhesion molecule-1 (ICAM-1), intercellular adhesion molecule-2 (ICAM-2), vascular cell adhesion molecule-1 (VCAM-1)]; and, finally transit of leucocytes between endothelial cells into the extracellular matrix is mediated by platelet-endothelial cell adhesion molecule-1 (PECAM-1) on both leucocytes and endothelial cells.

2. Objectives

There are two objectives to our study:

- 1 to determine if Castanospermine impairs the binding between mononuclear cells and vascular endothelial cells
- 2 to determine if it changes the membrane expression of adhesion molecules on mononuclear cells or vascular endothelial cells.

3. Materials and methods

3.1. Castanospermine

This alkaloid was derived from the seeds of *Castanospermum australe* (the Australian Moreton Bay Chestnut) by a standard technique yielding purity $\geq 99.5\%$ [14]. Castanospermine was dissolved in Aim-V medium (Gibco, Mulgrave, Victoria, Australia) or Medium 199 complete (Gibco) to a concentration of $65,536 \mu\text{M}$ and then filtered through a $0.22 \mu\text{m}$ filter (Sartorius, Hannover, Germany). Using quadrupling dilutions a range of concentrations from $16,384 \mu\text{M}$ to $0.25 \mu\text{M}$ was prepared.

3.2. Monoclonal antibodies and fluorescent markers

Monoclonal antibodies (mAb) against the following cell adhesion molecules on peripheral blood mononuclear cells (PBMCs) were used: CD3

fluorescein isothiocyanate conjugated (FITC conjugated) (clone: HIT3a) (Pharmingen, BD Biosciences, Franklin Lakes, New Jersey, USA); CD 45 R-phycerythrin conjugated (PE conjugated) (clone: HI30) (Pharmingen); CD91 (PE conjugated) (clone: G155-228) (Pharmingen); CD11a (LFA1 α) (PE conjugated) (clone: HI111) (Pharmingen); CD11b (Mac-1) (APC conjugated) (clone: ICRF44) (Pharmingen); CD11c (CR4) (APC conjugated) (clone: B-ly6) (Pharmingen); CD49d (VLA-4) Allophycocyanin conjugated (APC conjugated) (clone: 9F10) (Pharmingen); CD62L (L-selectin) (APC conjugated) (clone: DREG-56) (Pharmingen); CD31 (PECAM-1) (PE conjugated) (clone: WM59) (Pharmingen); CD15s (Sialyl Lewis X) (clone: CSLEX1) (Pharmingen); and, CD162 (P-selectin glycoprotein ligand-1) (PSGL-1) (PE conjugated) (clone: KP-1) (Pharmingen). The secondary fluorescent marker used to label non-conjugated antibodies was F(ab')₂ rabbit anti mouse IgM (PE conjugated) (clone: R6-60.2) (Pharmingen). Monoclonal antibodies against the following adhesion molecules on human umbilical vein endothelial cells (HUVECs) were used: CD54 (ICAM-1) (clone: HA58) (Pharmingen); CD102 RPE conjugated (ICAM-2) (clone: CBR-1C2/2) (Pharmingen); CD106 (VCAM-1) (clone: 51-10C9) (Pharmingen); CD31 (PECAM-1) (PE conjugated) (clone: WM59) (Pharmingen); CD62E (E-selectin) (clone: 68-5H11) (Pharmingen); and, CD62P (P-selectin) (PE conjugated) (clone: AK-4) (Pharmingen). The secondary fluorescent marker used to label non-conjugated antibodies was goat anti mouse IgG/IgM (FITC conjugated) (polyclonal) (Pharmingen). These molecules were selected to represent the sequence of CAMs required for leucocyte binding to and transit between endothelial cells.

3.3. Preparation of peripheral blood mononuclear cells

They were prepared from venous blood of healthy donors using a standard technique [15].

3.4. Preparation of human umbilical vein endothelial cells

Human umbilical cords were obtained from deliveries at the birthing centre John Hunter Hospital (Newcastle, Australia) and collected into sterile Scotts bottles containing Hanks buffered salt solution (HBSS) (Invitrogen, Mulgrave, Victoria, Australia) supplemented with 75 U/ml gentamicin (Invitrogen) and $2.5 \mu\text{g/ml}$ fungizone (Invitrogen). For this project ethical approval was granted by the Human Research Ethics Committee of the Hunter New England Area Health Service, New South Wales, Australia (numbers 9712103.24 and 11/06/15/5.08). Donors gave written informed consent before umbilical cords were accepted. Human umbilical vein endothelial cells (HUVECs) were recovered from umbilical cords by a modification of the method described by Jaffe [16]. The purity of HUVECs was ascertained by their cobblestone morphology, their positive staining for CD31 and Von Willebrand Factor using Sheep Anti Human FITC conjugated mAb to Von Willebrand Factor (Dako, Glostrup, Denmark) and their CD144 membrane expression measured by flow cytometry using a fluorescence activated cell sorter (FACS) Canto (BD Biosciences Franklin Lakes, New Jersey, USA) and the BD FACS Diva software package (BD Biosciences). Human umbilical vein endothelial cells were cryopreserved for future use in 10% DMSO (BDH Chemicals, Australia Pty. Ltd, Kilsyth, Victoria, Australia) in FBS using the standard technique for cryopreservation. They were stored in liquid nitrogen and used from passages three to six.

3.5. Mononuclear/endothelial cell binding assay

It was done according to the procedure described by Cavender et al. [17]. For assays requiring treated HUVECs, $100 \mu\text{l}$ of CAST in M199 complete in a range of concentrations from $16,384 \mu\text{M}$ to $0.25 \mu\text{M}$ (in quadrupling dilutions) was added. For assays requiring non-treated HUVECs, $100 \mu\text{l}$ of M199 complete was added to each well instead. All assays were performed in triplicate. After 16 h HUVECs for both assays were stimulated with tumour necrosis factor

Table 1

Change in the percentage mononuclear/endothelial cell binding associated with an increase in the natural logarithm of concentration of Castanospermine.

Group ^a	PBMCs ^b	HUVECs ^c	Change	p-Value	Interaction ^d
1	Not treated	Dose response	−0.0001	0.978	0.001
2	Single dose	Dose response	−0.0221	<0.001	
3	Dose response	Not treated	−0.0059	0.582	0.154
4	Dose response	Single dose	−0.0261	0.004	

^a There are 5 experiments in each group.

^b Peripheral blood mononuclear cells.

^c Human umbilical vein endothelial cells.

^d Interaction between groups 1 and 2 or groups 3 and 4.

alpha (TNF α) (Peprotech, Rockhill, New Jersey, USA) at 10 ng/ml for 24 h. For assays requiring treated PBMCs, CAST in a range of concentrations from 16,384 μ M to 0.25 μ M (in quadrupling dilutions) was added to each well. After 12 h, these PBMCs were stimulated with interleukin 2 (IL-2) (Peprotech, Rockhill, New Jersey, USA) at 10 ng/ml for 48 h. For assays requiring non-CAST treated PBMCs, IL-2 was added after 12 h of incubation. PBMCs were then washed twice with HBSS plus 5% FBS and suspended at a cell concentration of 1×10^7 /ml in HBSS plus 5% FBS and labelled with Na 51 CrO $_4$ (GE Healthcare, Amersham, UK) at 200 μ Ci/ml for 60 min at 37 °C. Aliquots of 10^5 chromium labelled PBMCs were then added to each well in triplicate. The binding assay was allowed to proceed for 60 min at 37 °C. The bound PBMCs were lysed with 200 μ l of 2% sodium dodecyl sulphate (Sigma Pharmaceutical, South Croydon, Victoria, Australia) and the cell lysates were collected into counting tubes. The radioactivity in each tube was measured by an auto gamma counting system (Packard Instrument Company, Meriden, Connecticut, USA). For each dose of CAST in each experiment the percentage of cell binding was calculated by dividing the mean of the radioactivity of the bound cells by the mean of the total radioactivity of the added cells multiplied by 100 where the mean was derived from three readings. There were five experiments in each study group. Hence the final value for each dose of CAST was the mean of the five experiments.

3.6. Flow cytometry

Peripheral blood mononuclear cells were cultured on 24 well plates in Aim-V media to a cell concentration of 1×10^7 /ml. Human umbilical vein endothelial cells were cultured on 6 well plates

Fig. 1. Plot of the percentage of mononuclear/endothelial cell binding versus the natural logarithm of the concentration of Castanospermine used to treat human umbilical vein endothelial cells. The upper curve represents the binding between HUVECs treated with a range of concentrations of Castanospermine and untreated PBMCs. The lower curve represents the binding between HUVECs treated with a range of concentrations of Castanospermine and PBMCs treated with Castanospermine at 1024 μ M.

Fig. 2. Plot of the percentage of mononuclear/endothelial cell binding versus the natural logarithm of the concentration of Castanospermine used to treat peripheral blood mononuclear cells. The upper curve represents the binding between PBMCs treated with a range of concentrations of Castanospermine and untreated HUVECs. The lower curve represents the binding between PBMCs treated with a range of concentrations of Castanospermine and HUVECs treated with Castanospermine at 1024 μ M.

(NUNC) in M199 complete at a concentration of 1×10^5 cells/ml. Castanospermine in a range of concentrations from 16,384 μ M to 0.25 μ M (in quadrupling dilutions) was then added to each well and the cells were incubated for 12 h. Peripheral blood mononuclear cells were stimulated with IL-2 at 10 ng/ml for 48 h for all CAMs studied. To achieve maximum membrane expression HUVECs were stimulated with TNF α at 10 ng/ml for 6 h for E-selectin, for 18 h for VCAM-1, for 18 h for CD31, for 24 h for ICAM-1 and for 24 h for ICAM-2. For maximum P-selectin expression HUVECs were stimulated with IL4 (Peprotech, Rocky Hill, New Jersey, USA) at 20 ng/ml for 48 h. The membrane expression of CAMs was measured by flow cytometry using FACS Canto for PBMCs for HUVECs. Briefly 1×10^6 PBMCs or 1×10^5 HUVECs were mixed with 20 μ l of relevant conjugated mAb or 100 μ l of 1/100 dilution of the relevant mAb and incubated in the dark for 30 min at 4 °C. Cells were washed twice with cold PBS and 1% FBS, 100 μ l of 1/100 dilution of secondary antibody conjugated to RPE was added to cells incubated with purified mAb and incubated in the dark for 30 min at 4 °C. Cells were then washed twice with cold 1% FBS in PBS and resuspended in 500 μ l 2% paraformaldehyde in PBS. There were 5000 to 10,000 events analysed for each sample. For each experiment the mean fluorescence intensity ratio (MFIR) was calculated by dividing the mean fluorescence intensity of the test antibody by the mean fluorescence intensity of the respective isotype control antibody. The percentage of positive cells was calculated by the formula [(number of positive cells – background)/(total number of cells – background)] $\times$ 100. Eight CAMs were tested on stimulated PBMCs and six CAMs were tested on stimulated HUVECs. The full details of the methods used in Sections 3.3 to 3.6 are listed in supplementary digital content number 1.

3.7. Experimental design

3.7.1. Mononuclear/endothelial cell binding assay

Four groups were studied: 1. Non-treated PBMCs and HUVECs treated with a range of doses of CAST; 2. PBMCs treated with a single dose of CAST and HUVECs treated with a range of doses of CAST; 3. PBMCs treated with a range of doses of CAST and non-treated HUVECs; and, 4. PBMCs treated with a range of doses of CAST and HUVECs treated with a single dose of CAST. There were five experiments in each group. For each experiment the controls were the binding of stimulated PBMCs and stimulated HUVECs without CAST treatment and background radioactivity.

Table 2

Change in mean fluorescence intensity ratio and change in percentage of positive cells expressing adhesion molecules on human umbilical vein endothelial cells treated with increasing concentrations of Castanospermine.

Adhesion molecule	Mean fluorescence intensity ratio ^a		Percentage of positive cells ^a	
	Change	p-Value	Change	p-Value
E-selectin	−0.06 (−0.11 to −0.02)	0.003	0.02 (−0.33 to 0.38)	0.898
P-selectin	0.02 (0.01 to 0.03)	<0.001	0.66 (0.43 to 0.89)	<0.001
ICAM-1	−3.42 (−5.24 to −1.59)	<0.001	0.02 (−0.00 to 0.03)	0.083
ICAM-2	−3.24 (−5.46 to −1.02)	0.004	0.01 (−0.00 to 0.03)	0.060
VCAM-1	0.02 (−0.04 to 0.08)	0.616	0.76 (0.21 to 1.32)	0.007
PECAM-1	−0.81 (−1.22 to −0.39)	<0.001	0.00 (−0.01 to 0.02)	0.893

^a There are 5 experiments for each cell adhesion molecule studied.

3.7.2. Flow cytometry

The CAMs assayed on PBMCs, lymphocytes and monocytes were L-selectin, Sialyl Lewis X, PSGL-1, LFA-1 α , VLA-4, PECAM-1, Mac-1 and CR4. For subset analyses CD45 was used to gate for PBMCs, CD3 for lymphocytes and CD 91 for monocytes [18]. The CAMs assayed on HUVECs were E-selectin, P-selectin, ICAM-1, ICAM-2, VCAM and PECAM-1. There were 5 experiments for each CAM. The controls for each CAM were its expression on non-stimulated PBMCs without CAST treatment or on non-stimulated HUVECs without CAST treatment, background expression and the respective isotype control.

3.8. Statistical methods

3.8.1. Analysis of mononuclear/endothelial cell binding

To examine whether there was a statistically significant change in the level of radioactivity with increasing concentration and to test if the change was consistent across the four groups, a generalised linear mixed model was fitted to the data. The outcome in the model was

the level of radio activity where the predictor variables were concentration and group with experiment included in the model as a random effect to control for differences in baseline level of radioactivity between the five experiments within each group. To test if the change in radioactivity was different between the four groups an interaction term of group by concentration was added to the model [19].

3.8.2. Analyses of expression of cell adhesion molecules

Generalised linear mixed models were used to determine whether or not there was a statistically significant change in the outcome associated with an increase in concentration of CAST. Individual models were fitted for each adhesion molecule with the outcome in the model being the percentage of positive cells or the mean fluorescence intensity ratio: the predictor variable was the concentration of CAST, included as order or concentration. Experimental number was included in the model as a random effect to control for differences in baseline level of the outcome between the different experiments for each adhesion molecule [19].

4. Results

4.1. Mononuclear/endothelial cell binding

The results are described in Table 1 and Figs. 1 and 2. There was a statistically significant reduction in binding with an increase in CAST treatment of HUVECs when PBMCs were treated ($p < 0.001$) but not when the PBMCs were untreated ($p = 0.978$): the significant reduction in binding started when the concentration of CAST was 1 μM . The difference in slopes was statistically significant ($p = 0.001$) (Table 1, Fig. 1). There was also a reduction in the binding with an increase in the concentration of CAST on PBMCs when the HUVECs were treated ($p = 0.004$) but not when the HUVECs were untreated ($p = 0.582$): the significant reduction in binding started when the concentration of CAST was 16,384 μM . The difference in slopes was not statistically significant ($p = 0.154$) (Table 2, Fig. 2). The binding at zero concentration of CAST for both HUVECs and PBMCs was 41% in Fig. 1 and 43% in Fig. 2 (not significantly different). When comparing the two figures at zero concentration of CAST the difference in binding between the two treatment groups was 8% in Fig. 1 but there was no difference in binding between the two treatment groups in Fig. 2: in the former only

Fig. 3. Graphs of the effect of Castanospermine upon the mean fluorescence intensity ratio and percentage cell positivity for six cell adhesion molecules expressed on human umbilical vein endothelial cells. It shows the mean fluorescence intensity ratios (left ordinate, blue line) and percentage of positive cells (right ordinate, red line) for E-selectin; P-selectin; ICAM-1; ICAM-2; VCAM-1; and, PECAM-1 expressed upon HUVECs. Each curve is the mean of five experiments. The scale of the left ordinate is selected to highlight the relevant change.

Fig. 4. a: A representative flow cytogram for ICAM-1. It shows a left shift in mean fluorescence intensity for ICAM-1 on human umbilical vein endothelial cells when treated with Castanospermine. b: A representative flow cytogram for VLA-4. It shows a left shift in mean fluorescence intensity for VLA-4 on peripheral blood mononuclear cells when treated with Castanospermine.

PBMCs were treated but in the latter only HUVECs were treated. Furthermore when comparing the two figures at the point where both HUVECs and PBMCs were treated with CAST at 1024 μ M the binding was 25% in Fig. 1 (the lower curve) but it was 43% in Fig. 2 (lower curve): this is significantly different ($p < 0.05$).

4.2. Membrane expression of cell adhesion molecules on human umbilical vein endothelial cells

The results are displayed in Table 2 and Fig. 3. There were significant reductions in the MFIRs with increasing concentrations of CAST for E selectin, ICAM-1, ICAM-2 and PECAM-1; VCAM-1 was similar but P-selectin was increased. There were also significant increases in the percentage of positive cells for P-selectin and VCAM-1. A

representative flow cytogram showing a left shift in MFIR for ICAM-1 on HUVECs from CAST treatment is displayed in Fig. 4a.

4.3. Membrane expression of cell adhesion molecules on peripheral blood mononuclear cells

The results are displayed in Table 3 and Figs. 5 and 6. There were significant reductions in MFIRs with increasing concentrations of CAST for L-selectin, LFA1 α , VLA-4, Mac-1 and CR4 but an increase for PSGL-1 and PECAM-1. The changes in percentage of cell positivity were in a similar direction. A representative flow cytogram showing a left shift in MFIR for VLA-4 on PBMCs from CAST treatment is displayed in Fig. 4b.

4.4. Membrane expression of cell adhesion molecules on the subset of lymphocytes

The results are displayed in Table 4 and Figs. 5 and 6. The pattern of changes in MFIRs with the lymphocyte subset was similar to that on PBMCs but the reductions in expression of L-selectin, Sialyl Lewis X, LFA-1 α and VLA-4 and the increment in expression of PECAM-1 were larger.

4.5. Membrane expression of cell adhesion molecules on the subset of monocytes

The results are displayed in Table 5 and Figs. 5 and 6. The pattern of changes in MFIRs with the monocyte subset was similar to that on PBMCs except that PECAM-1 expression was reduced on monocytes. Further the reductions in expression of Mac-1 and CR4 were larger.

The PBMC subset analysis has shown that without CAST treatment the MFIR for LFA-1 α on lymphocytes was 123 and on monocytes was 38. Further the MFIR for VLA-4 on lymphocytes was 28 and on monocytes was 9.

5. Discussion

In order to achieve a reduction in mononuclear/endothelial cell binding it was necessary to treat both cell types with CAST which has also been shown to reduce the expression of multiple adhesion molecules on both cell types. There was, however, a difference in binding in Fig. 1 compared with Fig. 2 where both HUVECs and PBMCs were treated with CAST at 1024 μ M. This effect may be explained by a reduced percentage of lymphocytes in the PBMCs used in Fig. 1 compared with Fig. 2. The key adhesion molecules in PBMCs effecting firm adhesion to endothelial cells are thought to be LFA-1 α and VLA-4 [13]. The MFIRs for both are more on lymphocytes than on monocytes (Section 4.5). Therefore the ability to reduce binding by CAST would be increased if the percentage of lymphocytes in the PBMCs was decreased thus reducing the load of these key adhesion molecules subject to CAST. It is also possible that this factor provides an explanation for the difference in the concentration of CAST required to achieve a significant reduction in binding in Fig. 1 compared with Fig. 2.

Upon HUVECs, PBMCs and its lymphocyte and monocyte subsets the predominate effect of CAST was a reduction in MFIRs for nine CAMs although MFIRs were increased for three CAMs. It also reduced the expression of CAMs in all three phases of mononuclear/endothelial cell adhesion (rolling, firm adhesion and transmigration). Further it markedly reduced the expression of LFA-1 α and VLA-4 on lymphocytes and ICAM-1 and ICAM-2 on HUVECs which are key determinants of firm adhesion [13]. These findings imply that the reduction in

Table 3

Change in mean fluorescence intensity ratio and change in percentage of positive cells expressing adhesion molecules on peripheral blood mononuclear cells treated with increasing concentrations of Castanospermine.

Adhesion molecule	Mean fluorescence intensity ratio ^a		Percentage of positive cells ^a	
	Change	p-Value	Change	p-Value
L-selectin	-0.41 (-0.64 to -0.17)	0.001	-0.15 (-0.40 to 0.10)	0.245
Sialyl Lewis X	-0.03 (-0.06 to 0.01)	0.140	-0.01 (-0.32 to 0.30)	0.950
PSGL-1	0.11 (0.07 to 0.15)	< 0.001	2.56 (1.87 to 3.26)	< 0.001
LFA-1	-2.17 (-2.60 to -1.74)	< 0.001	-0.02 (-0.06 to 0.02)	0.435
VLA-4	-1.19 (-1.43 to -0.95)	< 0.001	-1.31 (-1.55 to -1.06)	< 0.001
PECAM-1	0.30 (0.02 to 0.59)	0.039	0.28 (0.12 to 0.45)	0.001
Mac-1	-0.16 (-0.24 to -0.07)	< 0.001	-0.28 (-0.54 to -0.01)	0.045
CR4	-0.75 (-1.00 to -0.50)	< 0.001	-0.21 (-0.46 to 0.05)	0.119

^a There are 5 experiments for each cell adhesion molecule studied.

Fig. 5. Graphs of the effect of Castanospermine upon the mean fluorescence intensity ratio for eight cell adhesion molecules expressed on peripheral blood mononuclear cells and their subsets of lymphocytes and monocytes. It shows the mean fluorescence intensity ratio for L-selectin; Sialyl Lewis X; P-selectin glycoprotein ligand; LFA-1α; Mac-1; CR4; VLA-4; and, PECAM-1, expressed upon PBMCs and subsets of lymphocytes and monocytes. Each curve is the mean of 5 experiments.

mononuclear/endothelial cell binding by CAST is due, at least in part, to decreased expression of CAMs on both cell types. By contrast Walter et al. [20] reported no effect of CAST on lymphocyte/endothelial cell binding or on the expression of LFA-1, ICAM-1 or VLA-4 on T cell lines. Our findings also help to explain its ability to control rejection established in several studies [5,6,21] by reducing endothelial cell binding and then cell entry into the allograft. It may also act by inhibiting heparanase and sulfatase secretion from endothelial cells thus inhibiting alloreactive cell transit in the graft [12]. Finally it is thought that CAST acts by decreasing T cell proliferation through inhibition of IL-2 signal transduction [20].

Castanospermine is a competitive inhibitor of glucosidase 1 and glucosidase 2 in the ER [9]. It therefore inhibits the first step in this process leading to high mannose containing oligosaccharides, impairment in transport through the ER and Golgi and membrane expression of N linked glycoproteins. Our study has shown that it impairs the expression of integrins (LFA-1α, VLA-4, Mac-1, CR4), members of the immunoglobulin supergene family (ICAM-1, ICAM-2, PECAM-1) and selectins (E-selectin). But the expression of some CAMs is unchanged and some are increased. The mechanism underlying this selective effect is poorly understood. It is thought that selective chains in heparan sulphate-glycosaminoglycan

(HS-GAG) are regulators of glycoprotein activity [22]. We postulate therefore that CAST interferes with the production of HS-GAG: this may then lead either to an increase or a decrease in the expression of cell adhesion molecules.

In the rat CAST is relatively non-toxic: after 3 and 7 days of treatment we found no significant changes in biochemical and haematological parameters but after 14 days of treatment there were mild elevation in some liver enzymes, reduction in bilirubin and reduction in red cell counts while other parameters were normal [5]. There was also no significant reduction in weight [5]. It also inhibits sucrase which may be the cause of diarrhoea at high doses [23]. In mice at high doses vacuolation in myocytes due to mannose rich oligosaccharides has been reported [24].

To date there has been limited application of immunosuppressive agents directed against CAMs in clinical transplant practice. Murine and humanised (efalizumab) anti LFA-1 mAbs have been used as induction agents in human renal transplantation [25,26]: although recipients experienced lower delayed graft function rates with the murine anti-LFA-1 mAb [25] recipients treated with higher dose of efalizumab (2 mg/kg) and full dose Cyclosporin A had a high incidence of post transplant lymphoproliferative disease [26]. Efalizumab

Fig. 6. Graphs of the effect of Castanospermine upon the percentage cell positivity for eight cell adhesion molecules expressed on PBMCs and their subsets of lymphocytes and monocytes. It shows the percentage of positive cells for L-selectin; Sialyl Lewis X; P-selectin glycoprotein ligand; LFA-1 α ; Mac-1; CR4; VLA-4; and, PECAM-1, expressed upon PBMCs and subsets of lymphocytes and monocytes. Each curve is the mean of 5 experiments.

combined with mycophenolic acid mofetil has also been used as a maintenance agent in human islet transplantation before its withdrawal from the market [27]. Furthermore in the European multicentre trial, induction with murine anti ICAM-1 mAb combined with a cyclosporin A based regime did not reduce acute rejection nor delayed graft function [28] but it did reduce delayed graft function in renal transplantation in another study [29]. Alefacept, a humanised LFA-3-IgG₁ fusion protein which blocks the binding between CD2

and LFA-3 has been shown to block human T cell memory responses including those resistant to belatacept [30]. Our findings may extend the use of agents against CAMs by blockade of binding between alloreactive cells and vascular endothelium within the allograft mediated by inhibition CAM expression.

Our study raises the possibility that a major site in the action of CAST is the endothelial cell [31] which to date has not been specifically targeted by immunosuppressants in transplantation. The suspicion

Table 4

Change in mean fluorescence intensity ratio and change in percentage of positive cells expressing adhesion molecules on lymphocytes treated with increasing concentrations of Castanospermine.

Adhesion molecule	Mean fluorescence intensity ratio ^a		Percentage of positive cells ^a	
	Change	p-Value	Change	p-Value
L-selectin	−1.93 (−2.78 to −1.07)	<0.001	0.03 (−0.17 to 0.23)	0.752
Sialyl Lewis X	−0.11 (−0.19 to −0.03)	0.005	−0.31 (−0.53 to −0.08)	0.008
PSGL-1	0.30 (0.21 to 0.39)	<0.001	2.71 (2.03 to 3.39)	<0.001
LFA-1	−5.74 (−6.97 to −4.52)	<0.001	0.02 (−0.01 to 0.06)	0.124
VLA-4	−3.98 (−5.07 to −2.91)	<0.001	−0.38 (−0.50 to −0.27)	<0.001
PECAM-1	1.54 (0.80 to 2.27)	<0.001	0.31 (0.12 to 0.50)	0.001
Mac-1	−0.03 (−0.07 to 0.01)	0.111	−0.26 (−0.46 to −0.05)	0.013
CR4	−0.14 (−0.19 to −0.08)	<0.001	−0.45 (−0.64 to −0.25)	<0.001

^a There are 5 experiments for each cell adhesion molecule studied.

Table 5
Change in mean fluorescence intensity ratio and change in percentage of positive cells expressing adhesion molecules on monocytes treated with increasing concentrations of Castanospermine.

Adhesion molecule	Mean fluorescence intensity ratio ^a		Percentage of positive cells ^a	
	Change	p-Value	Change	p-Value
L-selectin	−0.22 (−0.36 to −0.09)	0.001	−1.11 (−1.68 to −0.55)	<0.001
Sialyl Lewis X	−0.04 (−0.13 to 0.06)	0.439	0.99 (0.33 to 1.65)	0.003
PSGL-1	0.11 (0.06 to 0.15)	<0.001	3.25 (2.23 to 4.26)	<0.001
LFA-1	−2.65 (−3.20 to −2.11)	<0.001	−0.00 (−0.08 to 0.07)	0.902
VLA-4	−0.86 (−0.98 to −0.73)	<0.001	−4.41 (−5.23 to −3.59)	<0.001
PECAM-1	−0.09 (−0.28 to 0.10)	0.357	1.82 (1.39 to 2.27)	<0.001
Mac-1	−0.46 (−0.63 to −0.29)	<0.001	0.45 (−0.10 to 1.00)	0.110
CR4	−2.19 (−2.60 to −1.77)	<0.001	−0.31 (−1.03 to 0.41)	0.403

^a There are 5 experiments for each cell adhesion molecule studied.

that CAST may act on the endothelial cell was raised by studies showing that in rats with EAE, cardiac allografts or antigen induced poly-arthritis treated with CAST there were accumulations of lymphoid cells about venules and capillaries and a sparing of infiltrate into the target organ itself [5,7,8]. Further Bartlett et al. [12] showed that CAST impaired the ability of endothelial cells to degrade the extracellular matrix due to the impairment of the expression of heparanase or sulphatase. Our study also shows that it impairs the expression of E-selectin, ICAM-1, ICAM-2 and PECAM-1 on endothelial cells which may reduce the entry of alloreactive cells into the graft and possibly alleviate ischaemia reperfusion injury known to be mediated by endothelial cell activation [10].

6. Conclusions

Our conclusions are that CAST impairs mononuclear/endothelial cell binding if and only if both cell types are treated and that it impairs the expression of multiple adhesion molecules on both cell types including their subsets of lymphocytes and monocytes. These findings help to explain its immunosuppressive effect in transplantation. Further studies about the ability of CAST to impair cell migration through the allograft are now required.

Disclosure

There is no conflict of interest declared by any author.

Contributions

ADH: experimental design, wrote the paper, and critical review of the paper.

PRT: critical review of the paper.

DAC: experimentation and critical review of the paper.

PM: statistical analysis, wrote the statistical section, and critical review of the paper.

WBC: pioneered the study of Castanospermine, experimental design, and critical review of the paper.

Acknowledgements

This project was made possible by a grant from Kiriwina Investments Limited which played no part in the design, conduct or analysis of the study. We wish to acknowledge the assistance of the deceased donor coordinators in the Newcastle Transplant Unit and the research nurse in the Division of Obstetrics, John Hunter Hospital in obtaining consent from expectant mothers for the donation of umbilical cords. We thank Stephen McNally for his skill in constructing the figures.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.trim.2012.05.002>.

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