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

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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-1α ($p<0.001$), 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-1α 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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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

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-1α, lymphocyte function associated antigen-1α; MAb, monoclonal antibody; MFTI, 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; TNFα, tumour necrosis factor alpha; VCAM-1, vascular cell adhesion molecule-1; VLA-4, very late antigen-4.

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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 ≥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 μM and then filtered through a 0.22 μm filter (Sartorius, Hannover, Germany). Using quadrupling dilutions a range of concentrations from 16,384 μM to 0.25 μM 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-phycoerythrin conjugated (PE conjugated) (clone: HI30) (Pharmingen); CD91 (PE conjugated) (clone: G155-228) (Pharmingen); CD11a (LFA1α) (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 μg/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 μl of CAST in M199 complete in a range of concentrations from 16,384 μM to 0.25 μM (in quadrupling dilutions) was added. For assays requiring non-treated HUVECs, 100 μl 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

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