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Demonstration of P-selectin expression and potential function in human corneal epithelial cells

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ABSTRACT

In response to an unexpected observation of apparent localisation by immunocytochemistry, we have investigated the potential expression and function of P-selectin (CD62P) in human corneal epithelial cells. The SV40 immortalised cell line, HCE-T (validated by STR profiling), along with multiple donor corneal-limbal tissue samples, were examined for P-selectin expression using a combination of immunocytochemistry, Western blotting, RT-PCR and immunohistochemistry. Potential expression of the major ligand for P-selectin (P-selectin glycoprotein ligand-1; PSGL-1; CD162) was also examined by immunocytochemistry and RT-PCR. A selective inhibitor of P-selectin-PSGL-1 binding (KF38789) was subsequently tested for effects on HCE-T cells using a cell culture gap-closure assay. HCE-T cells as well as primary epithelial cultures derived from donor corneal-limbal tissue, displayed positive immunostaining for P-selectin. Staining was particularly evident at cell-cell boundaries and at the outer edge of expanding epithelial islands. P-selectin expression was confirmed by Western blotting and RT-PCR (validated by product sequencing), as well as by immunohistochemistry performed on serial sections of corneal-limbal tissue stained for P-selectin, keratin 3 and p63. PSGL-1 was detected by RT-PCR and immunocytochemistry in both corneal epithelial cells as well as human limbal fibroblasts (HLF). KF38789 (5 μM) significantly reduced closure of a 500-μm gap between confluent sheets of HCE-T cells over an 8-hr period (by ~40%, $p < 0.01$; paired two-tailed T test), but had no effect on culture gap-closure by either HLF or murine 3T3 fibroblasts. These results provide evidence of P-selectin expression in human corneal epithelial cells and suggest a potential role for this glycoprotein in facilitating the net movement of confluent sheets of human corneal epithelial cells.

1. Introduction

P-selectin (CD62P) is a key mediator of leukocyte recruitment during inflammation and wound healing (Ley, 2003). The established sources of P-selectin are the α-granules of platelets (Stenberg et al., 1985) and the Weibel-Palade bodies of vascular endothelial cells (McEver et al., 1989). During inflammation, P-selectin is translocated to the cell surface where it encourages the adhesion of leukocytes via binding to P-selectin glycoprotein ligand-1 (PSGL-1/CD162) (Bonfanti et al., 1989; Larsen et al., 1989). Soluble forms of P-selectin and PSGL-1 also exist and have been implicated in the development of numerous cardiovascular and inflammatory diseases (Ley, 2003). In the present study, however, we explore a previously undocumented source of this protein – the corneal epithelium.

The corneal epithelium is a non-keratinized, stratified squamous epithelium that forms the anterior surface of the cornea. Beneath the corneal epithelium resides a non-vascular corneal stroma populated predominantly by a resident mesenchymal cell type known as the keratocyte. During tissue homeostasis, the corneal epithelium is replenished through the proliferation, migration and differentiation of epithelial progenitor cells that, in humans, principally reside within the vascularised corneal-scleral junction known as the corneal limbus (Schermer et al., 1986; Thoft and Friend, 1983). Wounding of the corneal epithelium triggers apoptosis of local keratocytes, conversion of

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adjacent keratocytes into wound repair fibroblasts, and recruitment of leukocytes from the limbal vasculature (Fini, 1999; Wilson, 2000). Significantly, it has been established through use of mouse models, that P-selectin derived from platelets facilitates the recruitment of leukocytes from limbal blood vessels (Lam et al., 2011). Moreover, platelet derived P-selectin has been linked to efficient healing of both the corneal epithelium and corneal stroma (Lam et al., 2015; Li et al., 2006). Importantly, there has been no evidence to suggest that either P-selectin or its principal ligand (PSGL-1) are produced by corneal cells.

We presently explore an unexpected observation of apparent P-selectin immuno-localisation in cultures of human corneal epithelial cells. Our initial observation is made using the SV-40 immortalised human corneal epithelial cell line, HCE-T. Given widespread problems associated with the misidentification of cell lines and poor specificity of commercial antibodies, we embarked upon a series of experiments aimed at confirming the nature of the cells being studied and the origin of P-selectin apparently present in our cultures. After an extensive analysis of corneal tissue and cultures acquired from over a dozen tissue donors, we present evidence of P-selectin expression within corneal epithelial cells and provide preliminary data in support of a potential new function for this protein.

2. Materials and methods

2.1. Sourcing, cultivation and validation of cell lines

The human corneal epithelial cell line HCE-T was acquired from the Riken Cell Bank (Cat. No. RCB2280. Lot No. 002) and maintained in serum-supplemented growth medium as described previously (Hogerheyde et al., 2013). A consistent source and batch of foetal bovine serum (FBS) was used throughout all studies (Hyclone Thermo Scientific; Cat. No. SH30084.03). An independent STR profile analysis of our working stocks performed by the Garvan Institute of Medical Research (Sydney, Australia) confirmed closest identity to two reference HCE-T cell lines (RCB1384 and RCB2280; Supplementary Data 1 and 2). The human dermal microvascular endothelial cell line HMEC-1 was acquired from Professor T.J. Lawley of the Centers for Disease Control (CDC; Atlanta GA, USA) with subsequent confirmation of identity performed on a sample of working stock cells by STR profiling (Garvan Institute of Medical Research; data not shown). The primate choroidal vascular endothelial cell line RF6A and human umbilical vein endothelial cells (HUVEC; as used in Supplementary Data 8) were acquired directly from the American Type Culture Collection (ATCC, CRL-1780) without further validation. Endothelial cells were maintained in MCDB-131 medium supplemented with 10% FBS, 2 mM L-glutamine, 10 ng/mL epidermal growth factor, 1 mg/mL hydrocortisone and antibiotics.

2.2. Immunocytochemistry (ICC)

Cultures were fixed for approximately 20-min in 10% (v/v) neutral buffered formalin. In supplementary experiments (Supplementary Data 6, 7 & 8), some cultures subsequently received two 5-min incubations at room temperature in phosphate buffered saline (PBS) containing 0.3% Triton-X100. Blocking was performed by incubation in PBS containing 2% (v/v) normal goat serum (NGS) for 30-min at 37 °C. All primary antibodies were obtained from commercial sources and used according to manufacturer's instructions (Table 1). Staining was performed for 1-hr at 37 °C in PBS supplemented with 1% normal goat serum. Positive staining results were routinely validated by omission of the primary antibody step, but an isotype control was also tested (Table 1; Supplementary Data 6 and 8). Some cultures were counterstained to display cell nuclei by incubation for 30-min in HEPES buffered saline (20 mM HEPES/0.85% w/v NaCl) containing 2 μg/mL Hoechst 33342 nuclear stain (Invitrogen/Molecular Probes, Cat. No. H1399). Imaging of stained cultures was performed as described previously (Gillies et al., 2015).

2.3. Establishment and cultivation of primary corneal-limbal epithelial cells (HLE)

Cultures of non-transformed human corneal epithelial cells were established from either enzymatically-dissociated samples of limbal epithelial cells grown in the presence of growth-arrested 3T3 cells as described previously (Ainscough et al., 2011), or from 2-mm diameter punch biopsies of intact corneal-limbus seeded face down into serum-treated culture dishes (explant method). Tissue derived from cadaveric donors was used in each case. Each tissue sample was acquired with donor consent and ethics approval (QUT HREC approval no. 0800000807) in the form of either surgical off-cuts (courtesy of Dr Andrew Apel and staff at Queensland Eye Hospital) or intact corneas that had been rejected for transplant (e.g. poor endothelial cell density). All tissue was supplied in eye bank storage medium (Optisol) and washed in three changes of Hank's buffered salt solution. All cultures were grown in the presence of the same source and batch of FBS (10%) as described above for use with cell lines.

2.4. Isolation and culture of human limbal fibroblasts

A previously established and characterised stock of human limbal fibroblasts (HLF) was utilised (Bray et al., 2014). These cells were maintained in DMEM/F12 + GlutaMAX (Invitrogen) supplemented with 10% FBS and antibiotics. Prior immunophenotyping of these cells by flow cytometry revealed > 95% positive staining for CD73, CD90 and CD105, and < 5% staining for CD34 and CD45 (Bray et al., 2014).

2.5. Detection of P-selectin by Western blotting

Total protein lysates were made using RIPA buffer (Pierce Biotechnology) and quantified using a Qubit Protein Assay before 30 μg protein was diluted in 4x Laemmli Sample buffer (BioRad) with 355 mM fresh β-mercaptoethanol for separation by SDS-PAGE (10% separating gel with 5% stack). Proteins were transferred to BioTrace™ NT nitrocellulose membrane. Membranes were blocked with 5% skim milk before incubation with primary antibodies to P-selectin (1/500; Table 1) or GAPDH (1/10,000, Abcam) and a peroxidase-conjugated secondary antibody (1/10,000, Pierce Biotechnology). Immunoreactivity was detected using SuperRX X-ray film (Fuji Film Corporation) with the Amersham™ ECL Plus Chemiluminescence kit (GE Healthcare).

2.6. RNA extraction and cDNA synthesis

RNA was extracted from cells using the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method. Briefly, cells were homogenised in TRIsure reagent (Bioline), chloroform was added, and the RNA component separated from the lysate by centrifugation. Total RNA was precipitated using isopropyl alcohol and the pellet washed with 75% ethanol. The RNA was resuspended in water and quantified in a Qubit 2.0 Fluorometer (Invitrogen) using a Qubit RNA HS Assay Kit (Invitrogen) according to the manufacturer's instructions. RNA (2 μg) from each sample was used to prepare cDNA by reverse transcription. RNA samples were incubated with dsDNAse (Thermo Scientific) to degrade any contaminating genomic DNA and cDNA generated using random hexamer primers and RevertAid Reverse Transcriptase (Thermo Scientific).

2.7. Reverse Transcription Polymerase Chain Reaction

Reverse transcription polymerase chain reaction (RT-PCR) was performed with undiluted cDNA samples using Taq DNA polymerase (Thermo Scientific). Forward and reverse primers (Table 2; GeneWorks)