Expression of CD34 and L-Selectin on Human Corneal Keratocytes

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PURPOSE. To investigate the expression of CD34, a hematopoietic stem cell marker and an adhesion molecule, and its ligand L-selectin in the human cornea.

METHODS. Seventeen normal adult human corneal specimens were studied by immunohistochemistry using a panel of monoclonal antibodies against all three classes of the hematopoietic stem cell marker CD34 and its ligand L-selectin. An additional six corneal specimens were used for protein extraction and analysis by Western blotting, using the CD34 and L-selectin antibodies. PCR was used to determine expression of mRNA for CD34 and L-selectin in the corneal specimens.

RESULTS. Only corneal keratocytes showed positive immunostaining for all three classes of CD34. Western blotting confirmed the expression of CD34 by these cells and mRNA expression for CD34 in the corneal stroma was demonstrated by PCR. For L-selectin, positive staining around keratocytes was noted on immunohistochemistry but L-selectin could not be detected either by Western blotting or PCR.

CONCLUSIONS. Normal human corneal keratocytes express all three classes of CD34. The expression of this adhesion molecule on corneal keratocytes suggests that it may have a role in keeping the keratocytes anchored in their microenvironment between the collagen lamellae. The positive staining for L-selectin found by immunohistochemistry but not by Western blotting or PCR would indicate the presence of either another ligand from the selectin family or a cross-reactive epitope on corneal keratocytes. (Invest Ophthalmol Vis Sci. 2003;44:4689–4692) DOI:10.1167/iovs.02-0999

The transparency of the normal human cornea is maintained by the regular arrangement of collagen fibers that are produced by keratocytes, which are stellate cells that occupy 2.5 to 5% of the stromal volume.1 Keratocytes are anchored within the corneal stroma by interactions between neighboring keratocyte processes and the surrounding extracellular matrix. Although various molecular factors have been identified on human stromal keratocytes,4–5 our knowledge of how keratocytes interact with the surrounding extracellular matrix, and remain anchored in their microenvironmental niche, is incomplete. We explored the possibility of a cell adhesion molecule CD34, and its ligand, L-selectin (CD62L), in fulfilling this role.

CD34 is a surface glycoprophophoprotein expressed on lymphohematopoietic stem cells, vascular endothelial cells, embryonic fibroblasts, and fibroblast-like dendritic cells in connective tissues.4–5 In 2001 we first reported the presence of CD34 on human keratocytes.6 Subsequently, Toti et al.7 reported the presence of CD34 by immunohistochemistry on keratocytes in normal and diseased corneas. In this article we describe our findings of CD34 expression on human keratocytes and our investigations in detecting its known definitive ligand, L-selectin.8

METHODS

Twenty-five normal human corneal specimens were obtained from cadaver donors. Twelve specimens were snap frozen in liquid nitrogen and five were fixed in 10% formalin before sectioning. A further eight fresh corneal specimens were used, four for protein extraction analysis by Western blotting and four for mRNA extraction and PCR. The research was conducted in accordance with the tenets of the Declaration of Helsinki and was approved by the local ethics committee board.

Primary Antibodies

Monoclonal antibodies against the three different classes of CD34 were used: class 1, clone Bi-3C5 (Zymed, Cambridge, UK); class 2, clone ICH-3 (from C. Kinnon, Institute of Child Health, London, UK); class 3, clone 581 (Insight Biotechnology, Middlesex, UK), and TUK3 (DAKO, Cambridge, UK). Antibodies against L-selectin, (CD62l-clone FMC46; Serotec, Oxford, UK; and CD62l-clone SK11; BD Biosciences, Pharmingen, Oxford, UK), were obtained.

Immunohistochemistry

Six-micron sections of the corneal specimens were stained with a three-step indirect alkaline phosphatase technique, using the antibodies against CD34 and L-selectin as described above. All antibodies were used in optimum dilutions, as determined by titration on positive controls. Fast red (DAKO, Cambridge, UK) was used as the chromogenic substrate in cryosections and diaminobenzidine tetrahydrochloride (Dako) in formalin-fixed sections.

Western Blotting

Protein was extracted from corneal epithelium, stroma, and endothelium separately in the presence of a lysis buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 1% nonidet P-40) and protease inhibitor. Specimens were then subjected to SDS PAGE under nonreducing conditions and placed in preformed 7% Tris-acetate gels (NuPAGE Gel; Invitrogen, Paisley, UK). Appropriate molecular weight markers (41 to 210 kDa, SeeBlue Plus 2; Invitrogen) were also placed in each gel. Gels were...
then prepared for Western blotting and transferred onto polyvinylidene difluoride (PVDF) membranes (Invitrogen). CD34 was identified by incubation with all three classes of primary mouse antihuman monoclonal antibodies. For L-selectin, the two mouse antihuman monoclonal antibodies, as described above, were used. Visualization of immunoreactivity was achieved using an immunodetection kit (Western Breeze; Invitrogen). Negative controls were performed on immunoblots incubated with nonrelevant isotype matched primary antibodies and visualized in a similar manner.

**Polymerase Chain Reaction**

RNA from epithelial and endothelial sheets was prepared using a proprietary kit (RNeasy, Qiagen Ltd, Crawley, West Sussex, UK) following manufacturer’s guidelines. RNA from stroma was prepared similarly, after grinding the tissue to powder in liquid nitrogen. Reverse transcription was performed on all RNA extracted using oligo(dT) primed first strand synthesis tubes (Ready-to-Go; Amersham Biosciences, Little Chalfont, Bucks, UK). Duplex PCR for CD34, L-selectin, and HPRT was performed on 1 ul of reverse transcription reaction in a 25-μl reaction using Taq enzyme and buffer (Invitrogen, Paisley, UK). The following primers were used for detection of CD34: CD34a 5′-GCAAGCCACACAGCTATCCAGGCTAT-3′; CD34b 5′-GGTCCAGGTTGTAAGCTATCCAGGCTAT-3′; for L-selectin (CD62L): CD62La 5′-TGTGTCACAGCAGGCTATCCAGGCTAT-3′; CD62Lb 5′-GACCAATGGAAGCTGAGGAA-3′; and for HPRT: HPRTa 5′-GACCAGTCAACAGGGGACAT-3′; HPRTb 5′-GACCTTGACCATCTTTGGA-3′. Cycle parameters were 10 minutes, 94°C (hot start), followed by 37 cycles (94°C, 1 minute; 54°C, 1 minute; 72°C, 1.5 minutes) and 72°C, 10 minutes to finish. Primers were at 0.2 μM each. PCR products were resolved on a 2% agarose gel (Invitrogen) according to the manufacturer’s instructions. HPRT (housekeeping gene control), CD34, and L-selectin PCRs produced 157 bp, 195 bp and 235 bp products, respectively.

**RESULTS**

Upon immunostaining, all specimens showed positive staining for CD34 on corneal stromal keratocytes. The staining was membranous and extended along the keratocyte processes (Fig. 1). There was no staining of any corneal epithelial cells (Fig. 1A) or endothelium (Fig. 1B). The same staining pattern was seen with all three different monoclonal antibody classes for CD34. No difference of any kind could be detected with use of different classes of antibodies. Positive staining for L-selectin was found around keratocytes and their processes (Figs. 2A, 2B), epithelium (Fig. 2A) and endothelium (Fig. 2B).

Western blotting for CD34 showed a distinct band at 110 to 120 kDa, which stained with all three classes of antibodies against CD34 (Fig. 3B). The epithelial and endothelial extracts did not show any positive staining for CD34 (Fig. 3C). PCR analysis of corneal tissue demonstrated mRNA expression for CD34 in the corneal stroma but not in the corneal epithelium or endothelium (Fig. 4). L-selectin could not be detected in the stroma, epithelium or endothelium by Western blotting or by PCR.

**Figure 1.** Photomicrograph of human cornea demonstrating (A) positive staining for CD34 class 1 (brown staining) on keratocytes in the corneal stroma (str) and absent staining in the epithelium (epi); and (B) no staining for CD34 class 1 on the corneal endothelium (endo). Magnification, ×400.

**Figure 2.** Photomicrograph of human cornea demonstrating (A) positive staining for L-selectin (pink staining) on epithelium (epi) and keratocytes in the stroma (str); (B) positive staining for L-selectin on corneal endothelium (endo); and (C) negative control showing absence of staining on corneal epithelium and stroma. Magnification, ×400.
binding proteins, nonmuscle myosin, and (NA), chymopapain (CP) and a glycoprotease (GP), have been epitopes, determined by enzyme sensitivity to neuraminidase like dendritic cells in connective tissues. Three distinct vessel endothelial cells, embryonic on early lymphohematopoietic stem and progenitor cells, small keratocyte adhesion with the extracellular matrix. CD34 is a have a limited knowledge of the molecular factors involved in calize with Connexin-43.9 Lauweryns et al. have shown expres-

FIGURE 4. Images of Western blot analysis of corneal tissue: (A) negative control (Negcon) using an irrelevant antibody (mouse anti-rat CD4) on corneal stromal tissue, (B) detection of expression of CD34 in corneal stromal tissue using class 2 anti-human CD34 antibody, and (C) absence of CD34 expression, using class 2 anti-human CD34 antibody in corneal epithelium (Epi) and corneal endothelium (Endo) tissue samples.

**DISCUSSION**

We have found that CD34 is strongly expressed by human keratocytes but not by the corneal epithelium or endothelium. The expression of CD34 was detected by immunohistochemistry; the presence of the protein and its mRNA was confirmed by Western blotting and PCR. We have also demonstrated positive staining with anti-L-selectin antibody around corneal keratocytes throughout the corneal stroma using immunohistochemistry. However, neither L-selectin nor its mRNA could be detected using immunoblotting or PCR.

Keratocyte studies using immunostaining have revealed that, at sites of cell–cell attachment, keratocytes express actin-binding proteins, nonmuscle myosin, and α-actin which colocalize with Connexin-43.5 Lauweryns et al. have shown expression of the beta 1 subunit of integrins but not alpha chains on stromal keratocytes.3 These findings were confirmed by Vorkauf et al. who have also described weak expression of ICAM 1 on normal keratocytes.2 However, at present we only have a limited knowledge of the molecular factors involved in keratocyte adhesion with the extracellular matrix. CD34 is a heavily glycosylated type 1 transmembrane protein expressed on early lymphohematopoietic stem and progenitor cells, small vessel endothelial cells, embryonic fibroblasts, and fibroblast-like dendritic cells in connective tissues. Three distinct epitopes, determined by enzyme sensitivity to neuraminidase (NA), chymopapain (CP) and a glycoprotease (GP), have been identified on CD34.4 The monoclonal antibodies reacting against these epitopes are designated Class I (sensitive to NA, CP and GP), Class II (sensitive to CP and GP but not NA), and Class III (insensitive to NA, CP and GP).4 Experiments in hematopoiesis have suggested that differential expression of these epitopes may have an implication on the fate of stem cells expression.10 However, we found that all the three classes of CD34 were strongly expressed by the corneal keratocytes without any specific difference between classes.

Studies on the function of CD34 suggest that it may play a role in cytoadhesion, and signaling related to differentiation and proliferation.11–15 In primitive fibroblast-like dendritic cells in tumors and healing wounds, the CD34 reactivity disappears as the cells attain a more differentiated actin positive myofibroblastic or collagenous synthetic phenotype.14 The presence of CD34 on keratocytes may indicate that they belong to a less-differentiated class of fibroblast-like dendritic cells. CD34 is also expressed on high endothelial venules (HEV) and is thought to play a role in leukocyte adhesion and homing during the inflammatory process via its ligand, L-selectin.5 HEVs are found in lymphoid organs and sites of chronic inflammation. They represent postcapillary venules with endothelium that demonstrate tall-cell morphology and are functionally specialized to facilitate the transendothelial migration of circulating T and B lymphocytes.8 L-selectin has been shown to bind to both GLYCAM-1 and CD34 from high-endothelial venule cells in lymph nodes. Several studies have suggested a role for L-selectin in homing of hematopoietic stem cells to the bone marrow after transplantation.15–17 In the corneal stroma, where the population of keratocytes is relatively static and there is little in the way of cell traffic, a different role for CD34, possibly that of cytoadhesion, is more likely. Given that L-selectin is to date the only known definitive ligand for CD34, it is reasonable to postulate that L-selectin serves as a ligand for CD34 in the corneal stroma. However, it is interesting that although we detected its presence by immunohistochemistry, we could not detect it by Western blotting or by PCR of corneal stromal mRNA. It is therefore tempting to postulate that another member of the selectin family, with a cross-
reactive epitope, may be serving as a ligand for CD34 in the corneal stroma.

Keratocytes originate from the second wave of neural crest-derived mesenchymal cells that enter the eye during embryonic development. As such they are neither stem cells nor of the vascular endothelial phenotype. The presence of CD34 on keratocytes would suggest that these cells are similar to the fibroblast-like dendritic cells of the dermis (which also express CD34) but are of a collagenous synthetic phenotype. It has been hypothesized that CD34 plays a role in stem/progenitor cell localization/adhesion in the bone marrow. Our findings of expression of all the three classes of CD34 on the keratocytes further support the view that this molecule has a role in keeping the keratocytes anchored in their micromic, between the collagen lamellae.

References