Distribution of Very Late Activation Integrins in the Human Cornea

An Immunohistochemical Study Using Monoclonal Antibodies

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There is growing evidence that cellular adhesion mechanisms characterized by cell–cell and cell–matrix interactions are a fundamental process in the immunobiology of the cornea. Interactions with various extracellular matrix components are mediated by the very late activation (VLA) subgroup of the integrin superfamily of adhesion molecules. The six different VLA dimers known thus far consist of a common β subunit and a variable α (α1 to α6) subunit. They serve as receptors for laminin (α3 and α6), collagen (α2 and α3), and fibronectin (α4 and α5). Using in situ immunohistochemistry and monoclonal antibodies, the distribution of the common β subunit and the variable α-chains of VLA molecules was studied in normal human cornea and in cases with scarring or subepithelial/retrocorneal fibrous tissue. Epithelial cells were VLA-β2 and VLA-α3, -α4, -α5, and -α6 positive. This is consistent with their intercellular adhesion and may aid in their attachment to the basement membrane which is composed of collagen, laminin, and fibronectin. Keratocytes in normal stroma expressed only the common β5 subunit and no detectable α-chains. In regions of scar or fibrous tissue, however, an upregulated expression of the α-chains was detected. The VLA-α1, -α2, -α3, -α4, and -α5 could be detected. The corneal endothelium showed a strikingly strong positivity for all VLA integrins. This suggests that these integrins may play a role in the normal function of corneal endothelial cells.

Adhesion molecules of the very late activation (VLA) family are part of the integrin superfamily which also includes the leukocyte integrins (LFA-1, Mac-1, and p 150, 95) and the GP11b/11la vitronectin receptor family. Integrins are noncovalently linked heterodimers at the cell surface with a unique subfamily β-chain and distinct α-chains. Functionally, some integrins, eg, VLA-5, are characterized by their capacity to interact with their ligands at sites containing the tripeptide sequence Arg-Gly-Asp (RGD). The β1-integrins (also the VLA protein family, so-called because the first two identified members appeared unusually late after in vitro activation of T-cells) show the typical structural composition of the integrin supergene family, ie, a shared β1-subunit (130 kilodaltons [kD]) and variable α-subunits (135–210 kD). Six different α-subunits (α1–α6) have been defined. The ligands for VLA-2, -3, -4, -5, and -6 have been identified as laminin (VLA-3 and -6), collagen (VLA-2 and -3), and fibronectin (VLA-4 and -5). The exact function of VLA-1 has not yet been clarified, but it may play a role as a laminin and collagen receptor.

Cell adhesion molecules in the human cornea may be involved in the adherence of the various cell types in the underlying stromal components. These adhesion molecules probably are important in the homeostasis of normal cornea and in the pathogenesis of various ocular disorders. We used immunohistochemical techniques with monoclonal antibodies specific for the β1-chain and the α-chains of VLA-1 to 6 to determine the expression of the VLA molecules in the human cornea.

Materials and Methods

Four human corneas were studied. Tissues were obtained during keratoplasty (cases 2 and 3) or enucleation for malignant melanoma (cases 1 and 4). The corneal specimens were cut transversally into three equal parts, snap frozen in liquid nitrogen-cooled isopentane, and stored at −75°C until used for immunohistochemistry.
For immunohistochemistry, serially cut frozen sections were dried overnight at room temperature, fixed in absolute acetone for 10 min, and stained with a three-step indirect immunoperoxidase using the anti-VLA monoclonal antibodies listed in Table 1. The secondary and tertiary steps consisted of peroxidase-conjugated rabbit anti-mouse and peroxidase-conjugated swine anti-rabbit immunoglobulins (Igs). The VLA-3 and -6 were demonstrated with a four-step unlabeled peroxidase-antiperoxidase (PAP) technique, using primary rat antibody 16 and GoH3, respectively, followed by rabbit anti-rat Igs, swine anti-rabbit Igs, and rabbit-PAP complex. All primary antibodies were used in optimal dilutions, determined by titration on positive controls. All secondary and tertiary antisera and the PAP complex were diluted in 10% human AB serum to reduce unwanted background staining. All samples were incubated for 30 min at room temperature, followed by a wash in three changes of PBS, pH 7.2. The reaction product was developed with the use of 3-amino-9-ethyl-carbazole substrate and H2O2 according to the method of Graham et al.14

Staining of the various components in the human cornea was graded semiquantitatively on a five-point scale: - , ±, +, ++, and ++++. Controls consisted of omission of the primary antibody or use of the chromogen alone.

**Results**

On routine histology, the two specimens obtained after enucleation (cases 1 and 4) contained central, peripheral, and limbal corneal tissue and appeared completely normal. Case 2 had bullous epithelial keratopathy with subepithelial young fibrous tissue invading by plump fibroblastic cells. Moreover, in the same case, a retrocorneal fibrous membrane could be seen between Descemet's membrane and the corneal endothelium (Fig. 2A) and retrocorneal fibrous membrane (Fig. 3A). In normal cornea, intense reactivity was found in corneal endothelial cells (Fig. 1A) and in limbal vascular endothelium. Stromal keratocytes in normal cornea were weakly positive (Fig. 1A) but showed strong 

| VLA-α1 | TS2/7 | M. E. Hemler (Boston, MA) |
| VLA-α2 | CLB-143 | E. Klein (Ulm, FRG) |
| VLA-α3 | J143 | M. E. Hemler (Boston, MA) |
| VLA-α4 | BSG10 | K. M. Yamada (NIH, Bethesda, MD) |
| VLA-α6 | GoH3 | AEGK von dem Borne (Amsterdam, The Netherlands) |
| VLA-β1 | 4B4 | Coulter Immunology (Hialeah, FL) |

Table 1. Monoclonal antibodies (Mcabs) used in this study

<table>
<thead>
<tr>
<th>Epithelium</th>
<th>Keratocytes</th>
<th>Endothelium</th>
<th>Vascular endothelium</th>
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<tbody>
<tr>
<td>VLA-α1</td>
<td>- (±)</td>
<td>-</td>
<td>++</td>
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<tr>
<td>VLA-α3</td>
<td>++</td>
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<td>VLA-α5</td>
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</tr>
<tr>
<td>VLA-β1</td>
<td>++*</td>
<td>±</td>
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Staining intensity: +++ (strong), ++ (moderate), + (slight), ± (occasional staining), - (no staining).

**Table 2. Distribution of VLA-integrins in human cornea**

Bowman's membrane, a calcific band keratopathy was present.

The results of the immunohistochemical staining are summarized in Table 2. The expression of the common VLA-β1 chain in the epithelium, keratocytes, and corneal endothelium is illustrated in Figures 1A-4A. In normal cornea, intense reactivity was found in corneal endothelial cells (Fig. 1A) and in limbal vascular endothelium. Stromal keratocytes in normal cornea were weakly positive (Fig. 1A) but showed strong α1-immunoreactivity in areas of subepithelial scar tissue (Fig. 2A) and retrocorneal fibrous membrane (Fig. 3A). On the epithelial cells of the normal cornea, β1-immunoreactivity was found both in the cytoplasm and on the basolateral cell membrane, its intensity decreasing from basal to superficial epithelial cells (Fig. 4A).

The staining results for the six α-chains revealed differential expression of these integrin molecules on the various components of the human cornea. Figures 1B-4B show the distribution of the α1-chain in human corneal tissue. Normal corneal endothelium (Fig. 1B) was strongly positive, whereas stromal keratocytes reacted very weakly. In regions of subepithelial scar tissue (Fig. 2B) and retrocorneal fibrous membrane (Fig. 3B), however, stromal cells expressed α1-chains. In normal cornea, the epithelium lacked α1-chain expression (Fig. 4B). It was weakly positive in areas of scar tissue (Fig. 2B).

Figures 1C-4C show the distribution of the α3-chain in human corneal tissue. Corneal endothelium (Fig. 1C) was strongly positive; stromal keratocytes were unreactive. In regions of retrocorneal (Fig. 3C) fibrous tissue, very weak immunoreactivity was found. Normal corneal epithelium strongly expressed the α3-chain (Fig. 4C), the intensity of which decreased from basal to superficial epithelial cells.

Figures 1D-4D illustrate the pattern of the α5-staining in human corneal tissue. Corneal endothelium (Fig. 1D) was strongly positive; keratocytes were vir-
Fig. 1. Staining of the endothelial cell layer (e) in the human cornea with monoclonal antibodies against VLA-β1 (A), VLA-α1 (B), VLA-α2 (C), VLA-α3 (D), and VLA-α5 (E). Corneal endothelial cells display strong immunoreactivity for all antibodies. Descemet membrane (d); stroma (s). Three-step indirect immunoperoxidase, counterstained with Harris' hematoxylin, original magnification x60.

Identification negative. In regions of subepithelial scar tissue (Fig. 2D) and retrocorneal fibrous membrane (Fig. 3D), however, the α3-chain was expressed by stromal spindled cells. Cells of the normal corneal epithelium displayed membranous reactivity for α3 (Fig. 4D). Identical staining patterns were obtained with anti-α6 monoclonal antibody.

Figures 1E–4E show the distribution of the α5-chain in human corneal tissue. Normal corneal endothelium (Fig. 1E) strongly expressed the α5-chain; stromal keratocytes were weakly negative. In areas of subepithelial (Fig. 2E) and subendothelial (Fig. 3E) scar tissue, however, upregulated α5-chain expression was observed. Display of the α5-chain in normal corneal epithelium (Fig. 4E) was most pronounced in the basal cell layer and decreased toward superficial layers. The distribution of the α6-chain was identical to that of α3 (Figs. 1C–4C), although there was no staining of the α6-chain on keratocytes in regions of fibrous tissue. Finally, the limbal vascular endothelium expressed all α-chains except α4 which could not be detected clearly.

Discussion
Using in situ immunohistochemistry and a panel of monoclonal antibodies, we investigated the distribu-
Fig. 2. Staining of the corneal epithelium (c) and subepithelial fibrous tissue (f) with monoclonal antibodies against VLA-β1 (A), VLA-α1 (B), VLA-α2 (C), VLA-α3 (D), and VLA-α5 (E). Corneal epithelial cells show immunoreactivity for VLA-β1, α2, α3, and α5, with more intensive staining basally than suprabasally. Keratocytes at the site of the subepithelial fibrous tissue express VLA-β1, α3, α5, and α6. Bowman’s membrane (b); stroma (s). Three-step indirect immunoperoxidase, counterstained with Harris’ hematoxylin, original magnification ×60.

tion of the VLA family of adhesion molecules in the human cornea. We found that corneal epithelium, endothelial cells, and keratocytes differentially express various members of the VLA integrins.

All epithelial cells were VLA-β1 and VLA-α2, α3, α4, α5, and α6 positive. The VLA-α3 (collagen receptor)3,11,12 and VLA-α4 (collagen, laminin, and fibronectin receptor)3 were more strongly expressed than VLA-α4 and α5 (fibronectin receptor)10,11,13 and VLA-α6 (laminin receptor).8 Moreover, basal cells expressed VLA-α3, α5, and α6 more strongly than suprabasal cell layers. Since laminin, collagen type IV, and fibronectin are intrinsic components of the basement membrane,15,16 it can be assumed that these integrins serve in the attachment of the epithelium to the basement membrane. In addition, localization of the integrins on the basolateral cell membranes is consistent with the concept that VLA molecules also play a role in intercellular adhesion.17 Migration and attachment of epithelial cells in epithelial wound healing presumably also involve such molecules with the formation and disruption of cell–cell and cell–matrix interactions.18 This point clearly needs further investigation.
Keratocytes in normal corneal stroma were found to lack VLA-α integrins, and only the common β₁-chain was weakly positive on these stromal cells. This finding can be explained by an “incoordinate” expression of α- and β-chains, the presence of a still unknown α-chain, or a progressive loss of α-chains in normal corneal stroma with its reappearance in an activated state. The latter possibility is supported by the immunohistochemical findings in case 3, where activated keratocytes in regions of scar tissue formation demonstrated positivity for VLA-α₁, -α₂, -α₄, and -α₅. Both VLA-4 and VLA-5 are considered to be fibronectin receptors.¹⁰,¹¹,¹³ VLA-3 being supposed to be a receptor for laminin, collagen, and fibronectin.³ The exact function of VLA-1 has not been clarified, but it probably serves as a laminin and collagen receptor. This “upregulated” expression of α-chains in activated keratocytes is consistent with the view that fibronectin plays an important role in wound healing, especially in young scar tissue. This is illustrated in the...
subepithelial fibrous tissue in case 2, where VLA-\(\alpha_4\) and \(\alpha_5\) were more strongly expressed than VLA-\(\alpha_1\), and \(\alpha_3\). In contrast, in regions of older and more organized fibrous tissue, as in the retrocorneal fibrous membrane, the keratocytes also displayed a strong reactivity for VLA-\(\alpha_1\), \(\alpha_2\), and \(\alpha_5\). Upregulation of these cellular receptors for collagen on keratocytes in retrocorneal fibrous membrane complies with the finding that these membranes\(^{19}\) are composed of fibrillar collagens, among which collagen type I secreted by its fibroblastic cells, is the predominant species.\(^{20,21}\)

The limbal vascular endothelium was found to express all VLA integrins, except the \(\alpha_4\)-chain which could not clearly be detected. This finding contrasted with earlier studies in the skin, in which vascular endothelium was found to lack VLA-\(\alpha_4\).\(^{22}\) This phenotype may render limbal vascular endothelium hitherto unknown functional properties.

The most striking observation in this study was the generalized and strong positivity of the corneal endothelium for all members of the VLA integrins. This phenomenon could be related to the strong adherence of the endothelial monolayer to the underlying Descemet's membrane made up of collagenous layers, laminin, and fibronectin and to the lifelong production of this membrane by the endothelial cells.\(^{23}\) Besides this cell-matrix adhesion, cell-cell interactions also
play an important role between the endothelial cells. The major function of the endothelium is to maintain corneal deturgescence through the action of specific pumps. For this function, a close adherence between individual cells is required. Our data suggest that the VLA family of integrins plays a role in the cellular adherence of corneal endothelial cells and thereby adds to their normal function.

Key words: VLA-integrin, immunohistochemistry, human cornea

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References


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