Distribution of integrins $\alpha_v\beta_5$, $\alpha_v\beta_3$ and α_v in normal human cornea: possible implications in clinical and therapeutic adenoviral infection

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ARTICLE

Abstract

Purpose **Integrins are heterodimeric cell surface molecules involved in cell–cell and cell–matrix interactions. Adenoviral entry into human cells has been shown to be dependent on integrins $\alpha_v\beta_5$ and $\alpha_v\beta_3$ that promote viral internalisation. We studied the distribution of integrins $\alpha_v\beta_5$, $\alpha_v\beta_3$ and the α_v chain in normal human cornea to investigate possible mechanisms of adenoviral entry to specific corneal cell types.**

Methods **We used immunohistochemistry with monoclonal antibodies to study the distribution of $\alpha_v\beta_5$, $\alpha_v\beta_3$ and α_v in normal human corneas maintained for up to 4 days in corneal storage medium (Optisol) at 4 °C ($n = 9$).**

Results **Both $\alpha_v\beta_5$ and α_v were present to a variable extent on the corneal epithelium and corneal endothelium of most specimens. In some specimens staining of both $\alpha_v\beta_5$ and α_v in the epithelium was graded, with more basal than superficial staining. $\alpha_v\beta_3$ was not detectable in either the corneal epithelium or the corneal endothelium in those specimens tested.**

Conclusions **The integrin $\alpha_v\beta_5$ is present on both epithelium and endothelium in the normal human cornea. The role of α_v integrins in clinical infection and in adenoviral entry for gene transfer is discussed.**

Key words Cornea, Integrin, Adenovirus, Gene therapy, Epithelium, Endothelium

Adhesion molecules are cell surface proteins that play an important role in cell–cell and cell–matrix interactions. There are at least four classes of adhesion molecules currently known: integrins, selectins, cadherins and the immunoglobulin superfamily.

Integrins

Integrins are transmembrane glycoproteins composed of two non-identical polypeptide chains – the α and β subunits – that are non-covalently associated. All α chains have a long extracellular domain and shorter intracellular domain, whereas β chains have long extracellular and intracellular domains. The β intracellular domain anchors the integrin heterodimer to the cytoskeleton. The α and β chains have multiple molecular variants; at least 14 distinct α chain and 8 β chain types have been described,[1] although new variants continue to be discovered. Several of the α and β chains form heterodimers with more than one complementary chain, although not all combinations are seen. The vitronectin receptor α_v can combine with at least five types of β chain.[2] In most cells the β chain is expressed in excess. As appearance of integrins on the cell surface depends on heterodimerisation, the expression of integrins on the cell surface seems to be determined by regulation of production of the α chain.[3] $\alpha_v\beta_5$ was first characterised in 1990,[4] and the role of the α_v integrins in adenoviral internalisation was elucidated in 1993.[5] α_v integrins have recently been demonstrated to play a role in angiogenesis, both in cornea and in retina.[6,7]

Adenovirus

Adenoviruses were first isolated in 1953.[8] They are non-enveloped double-stranded DNA virions, approximately 60–90 nm in diameter, comprising 87% protein and 13% DNA. The viral capsid has a rod-like fibre protein at each of its 12 vertices, giving it a characteristic appearance. Human adenoviruses have been divided into at least six groups and there are at least 47 different serotypes.[9]

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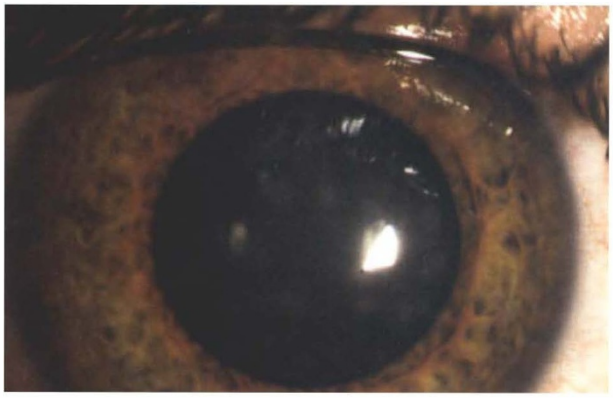

Fig. 1. *Adenoviral keratitis showing subepithelial corneal infiltrates.*

Adenoviral entry into cells is important clinically in the aetiology of adenoviral keratoconjunctivitis. It has been shown to involve two separate stages: firstly viral attachment via a recently elucidated fibril receptor,[10] and secondly viral internalisation via α_v integrins. It is thought that the peptide sequence RGD (Arg–Gly–Asp), which is found both in vitronectin and in the penton base of the adenoviral coat protein, mediates this internalisation.[5] Many serotypes including the clinically important serotypes 3 and 4 have been shown to use α_v integrins for transfection.[11,12]

Clinical infection

Adenoviral keratoconjunctivitis (Fig. 1) is common and accounts for significant morbidity. A broad range of serotypes can cause infection, commonly 3, 4, 7, 8 and 19. The typical presentation is acute onset of fever, pharyngitis, epiphora and papillary and follicular conjunctivitis. If the cornea is affected, punctate epithelial erosions are typical and cause foreign body sensation. Superficial stromal opacities develop later and these may take weeks, months or even years to resolve. There is at present no effective antiviral agent available for treatment of ocular adenoviral infection.

Adenovirus as a vector in gene therapy

Recombinant forms of human adenoviruses are being evaluated as vectors for gene transfer in many clinical trials. These adenoviruses are rendered replication-deficient by removing genes necessary for viral replication, while retaining those for cellular entry and gene expression.

In the eye, transfer of marker genes to ocular tissues has been demonstrated by several investigators. In the anterior segment gene transfer to mice, rat and rabbit cornea *in vivo* and human cornea *in vitro* has been shown[13–16] and the production of putative therapeutic genes has been demonstrated.[15,17] Most studies have shown expression of transfected gene preferentially in corneal endothelial cells, with much lower levels of recombinant protein production in corneal epithelium.

In gene therapy trials in cystic fibrosis, it has been shown that expression of $\alpha_v\beta_5$ integrin is necessary for efficient adenovirus-mediated gene transfer to the human airway.[18] Furthermore adenoviral gene transfer in the lung occurs more efficiently in distal airway epithelium where there is higher expression of $\alpha_v\beta_5$ integrin than in nasal epithelium, and this transaction can be blocked by RGD peptides.[19] Expression of $\alpha_v\beta_5$ in the eye may also be of importance in this context, in that specific blocking by RGD peptides might be envisaged.

Methods

Nine normal human corneas that were unsuitable for transplantation were obtained from the eye bank. They had been stored in Optisol (Chiron) at 4 °C for between 1 and 4 days before use. The corneal specimens were cut in half, snap-frozen in a minimal volume of normal saline in liquid nitrogen and then stored at −70 °C until sectioned.

For immunohistochemistry, 6 μm serially cut, frozen cryostat sections were placed on gelatin-coated slides and dried for a minimum of 2 h or overnight at room temperature. They were then fixed in absolute acetone for 10 min and air dried before wrapping in aluminium foil for storage at −20 °C until staining.

A two-step indirect immunoperoxidase technique was used, as follows. Cryostat sections were warmed slowly to room temperature and placed in a moist chamber. Sections were rehydrated with phosphate-buffered saline (PBS) and then 50 μl of the primary monoclonal antibody, a mouse anti-human integrin (Table 1), was applied over the specimen for an incubation period of 30 min at room temperature. The dilution was optimised to give the clearest staining results – approximately 5 μg/ml. The $\alpha_v\beta_5$ monoclonal antibody (Gibco Life Technologies) was used at a dilution of 1 in 50; the monoclonal antibodies to α_v and $\alpha_v\beta_3$ were used as undiluted hybridoma supernatant. After incubation the slides were washed twice with PBS and the second-layer antibody, 50 μl rabbit anti-mouse Ig peroxidase (Dakopatts) at a dilution of 1 in 100, was added for an incubation period of 30 min. This layer was diluted in PBS containing 5% normal human serum, which reduces background staining caused by antibody cross-reaction with human immunoglobulins in the tissue. Sections were then washed twice in PBS and incubated for 10 min with 50 μl of the substrate diaminobenzidine (DAB) at

Table 1. *Anti-integrin monoclonal antibodies used in this study*

Clone	Specificity	Dilution	Source
P1F6	$\alpha_v\beta_5$	1:50	Life Technologies, Middlesex, UK
23C6	$\alpha_v\beta_3$	Undiluted	Gift from Prof. Horton, UCL, UK
13C2	α_v	Undiluted	Gift from Prof. Horton, UCL, UK

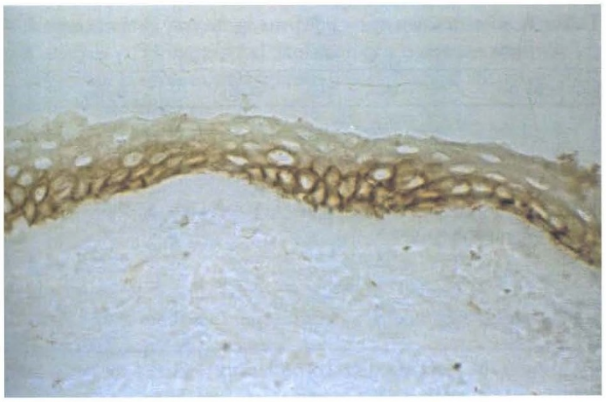

Fig. 2. *Immunoperoxidase staining of cornea (×100) showing moderate (++) corneal epithelial staining for the α_v integrin subunit.*

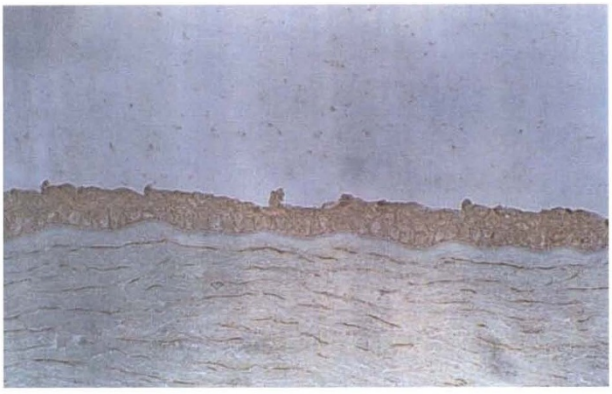

Fig. 4. *Immunoperoxidase staining of cornea (×100) showing moderate (++) corneal epithelial staining for the $\alpha_v\beta_5$ integrin heterodimer.*

0.6 μg/ml in PBS. Finally sections were washed twice with PBS and mounted in Kaiser's water-based mountant.

Results

All corneas showed positive corneal epithelial staining for α_v and in three of these corneas the epithelial staining was graded, with stronger staining basally than superficially, as shown in Fig. 2. Seven of the nine showed positive epithelial staining for $\alpha_v\beta_5$, although this was weaker than the α_v staining (Fig. 4, Table 2). No cornea showed $\alpha_v\beta_3$ staining (Fig. 6). All nine corneas showed positive corneal endothelial staining for α_v and $\alpha_v\beta_5$, as shown by Figs. 3 and 5 and Table 3.

Controls, which were consistently negative, consisted of substitution of primary antibody by PBS, omission of peroxidase-conjugated antibodies, or use of chromogen alone. A positive control of human breast carcinoma was used for the $\alpha_v\beta_3$ antibody, which showed clear staining of vascular endothelial cells.

Discussion

Using immunohistochemistry and a panel of three monoclonal antibodies to integrins $\alpha_v\beta_5$, $\alpha_v\beta_3$ and the α_v chain, we studied the distribution of these adhesion molecules in normal human cornea. Both $\alpha_v\beta_5$ and the α_v chain are present on the epithelium and endothelium at detectable levels, but if any $\alpha_v\beta_3$ integrin is present it is below the levels of detection using these techniques.

Several other studies have examined the expression of integrins in the human cornea,[20–23] although not $\alpha_v\beta_5$. Lauweryns *et al.*[22] found occasional faint granular staining in the corneal epithelium with α_v but not $\alpha_v\beta_3$ and suggested that the α_v chain in cornea was linked to a different β chain. We propose that this could be β_5. Tervo *et al.*[20] demonstrated faint basal staining of the normal corneal epithelium with a monoclonal antibody to α_v, which is consistent with our findings. All three authors frequently found a gradation of staining in the normal corneal epithelium for various adhesion molecules, with stronger staining basally and weaker staining nearer the corneal surface. This was apparent in some, but not all of our normal specimens. In the rat, Stepp *et al.*[24,25] demonstrated the presence of α_v, β_3 and β_5 subunits in the corneal epithelium and demonstrated upregulation of β_4 after corneal epithelial injury. Latvala *et al.*[26] showed upregulation of the α_6 chain after epithelial abrasion in the rabbit. It may be that damage to the corneal epithelium causes upregulation of α_v integrins and hence increased susceptibility to adenoviral infection.

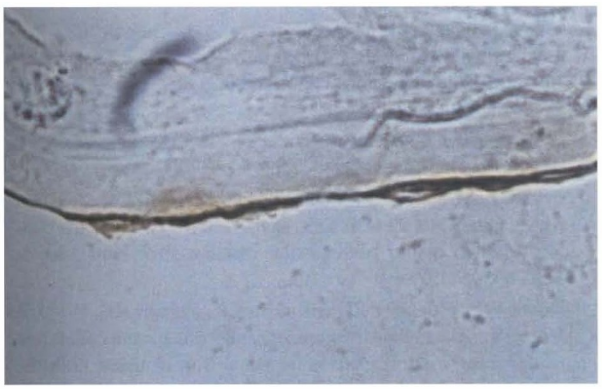

Fig. 3. *Immunoperoxidase staining of cornea (×400) showing moderate (++) corneal endothelial staining for the α_v integrin subunit.*

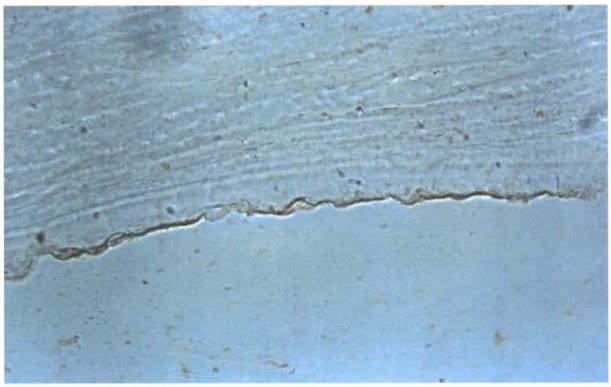

Fig. 5. *Immunoperoxidase staining of cornea (×200) showing moderate (++) corneal endothelial staining for the $\alpha_v\beta_5$ integrin heterodimer.*

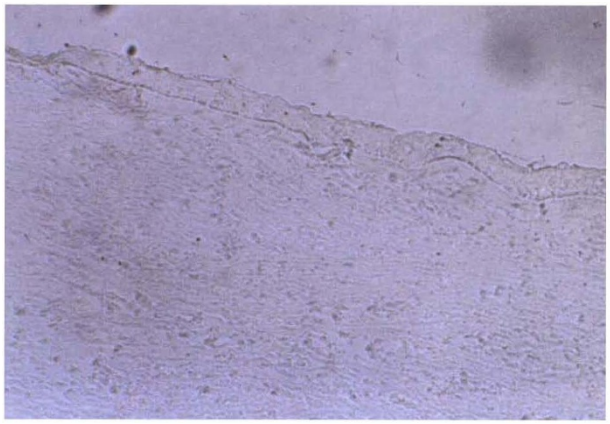

Fig. 6. *Normal cornea (×100) showing no staining with the $\alpha_v\beta_3$ integrin heterodimer.*

Another reason for interest in the distribution of these integrin molecules and the α_v subunit arises from the observation that after transfection by recombinant adenoviruses, marker gene expression is largely restricted to corneal endothelial cells and only occasionally seen in corneal epithelial cells. Often the few epithelial cells that express transfected gene product are near a cut edge of cornea.[12] However, a study of adenovirus-mediated gene transfer to rabbit by Abraham *et al.*[15] in 1995 of a putative therapeutic gene, haem oxygenase-1, detected transfected mRNA in both corneal epithelial and endothelial cells.

Therefore the detection of $\alpha_v\beta_5$ and α_v on both epithelial and endothelial cells in normal cornea, coupled with the observation that transfected mRNA is demonstrable in both layers, suggests that viral entry may occur in both cell types. If this is the case, the observed difference in transgene expression might be at the level of translation. Endothelial cells are metabolically more active than epithelial cells, which may be one explanation for this observed difference in transgene expression. An alternative explanation might be that a physical barrier of mucus or other ocular surface components prevents viral access, despite the presence of internalisation integrins. A third important point focuses on the physiological proliferation of corneal epithelial cells. If a transfection is not effective in limbal stem cells, transfected gene expression will only be effective for a limited short time period.

Table 2. *Distribution of α_v integrins in normal human corneal epithelium*

Cornea no.	α_v	$\alpha_v\beta_3$	$\alpha_v\beta_5$
1	+	−	+
2	++	−	++
3	+	−	+/−
4	+	−	+
5	+/−	−	−
6	++	−	+/−
7	++	−	+/−
8	+	−	−
9	++	−	++

++, moderate staining; +, faint staining; +/−, few cells, stain faintly; −, no stain.

Table 3. *Distribution of α_v integrins in normal human corneal endothelium*

Corneal no.	α_v	$\alpha_v\beta_3$	$\alpha_v\beta_5$
1	++	−	++
2	+/−	−	+/−
3	++	−	++
4	+/−	−	+/−
5	++	−	++
6	+/−	−	++
7	++	−	++
8	++	−	++
9	++	−	++

++, moderate staining; +, faint staining; +/−, few cells, stain faintly; −, no stain.

Integrins are an important group of adhesion molecules in the cornea. We have demonstrated that the integrin $\alpha_v\beta_5$ is expressed in both epithelium and endothelium in a panel of nine normal human corneas stored in Optisol for up to 4 days. However, levels of expression may be altered by the storage conditions. This finding could have relevance to clinical and possible therapeutic adenoviral entry to corneal epithelium and endothelium. The possibility of blocking adenoviral entry using monoclonal antibodies or RGD peptides raises interesting possibilities for future therapeutic strategies in clinical infection.

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