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Review

Corneal integrins and their functions

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Abstract

Integrins were first described just over 20 years ago and have been studied in the cornea by many groups interested in how the cornea functions in health and disease. There are a minimum of 12 different integrin heterodimers reported to be expressed by the major resident cells of the cornea: the corneal and limbal epithelial cells, keratocytes/fibroblasts, and corneal endothelial cells. These different integrin heterodimers play important and varied roles in maintaining the cornea and organizing how its cells interact with their surrounding extracellular matrix to maintain corneal clarity. In this review, an overview of the discovery and functions of integrins is provided along with a description of the current state of our knowledge of this large family of important proteins. While we have learned a lot about corneal integrins over the past 20 years, there is still much to learn. Areas where gaps in our knowledge of integrin functions in the cornea are slowing our progress in understanding corneal diseases and dystrophies at a molecular level are highlighted.

Keywords: cornea; matrix; cell adhesion; wound healing; integrins

1. Overview

The cornea consists of three tissue layers: the corneal endothelium, the stromal fibroblasts also called keratocytes, and the corneal epithelium. These layers are separated from one another by specialized basal laminae. The corneal endothelial cells sit on Descemet's membrane whereas the corneal epithelial cells sit upon the epithelial basement membrane. Subjacent to this basement membrane is an acellular zone, which is called Bowman's Layer in humans, primates, and birds. The corneal epithelial cells of many mammals including rats and mice lack a distinct Bowman's layer but never the less, their epithelial cells maintain adhesion to an underlying basement membrane zone containing laminins and collagens and exhibiting typical morphological features common to all basement membranes. While the three cells types described above are considered the major resident cells present in the cornea, additional cells can be found in the healthy cornea. These come in two major classes: immune cells, often called dendritic cells, and neuroglial (Schwann) cells found around bundles of axons that invest the cornea. While both immune and neural-derived cells likely play important roles in maintaining corneal health, little is known about their expression of integrins in the cornea.

Work conducted in the late 1970s suggested that the surfaces of cancer cells were different from normal cells and that factors in the serum, what later became known as growth factors, were able to interact with the surfaces of cancer cells differently than with normal cells. Studies conducted to sort out what these cell surface differences lead to the discovery of the integrin family of receptors. Along the way, important insights were gained into the roles played by various adhesive glycoproteins secreted by cells including fibronectin, vitronectin, and tenascin in cancer, immunology, and development (Hynes, 2004). By the mid-1980s several lines of evidence pointed to the existence of a family of integral membrane glycoproteins expressed on most of the cells in the body that function to mediate cell attachment to various glycoproteins

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including fibronectin and vitronectin but extending to include various laminins and collagens. These proteins were observed to sense changes in their extracellular environment and somehow transmit that information to the nucleus via alterations in the organization of the cytoskeleton (reviewed in ffrench-Constant and Colognato, 2004; Danen, 2005).

Integrins function as heterodimers consisting of individual α and β chains. They can be classified into three major groups: β1, β2, and αv. The β1 family consists of the first integrin to be discovered, β1, which can form heterodimers with at least 12 distinct α chains. The first six α chains had been identified as the VLA (very late after) activation antigens 1–6 and were shown to form heterodimers with the common β1 subunit. The VLA antigens were referred to as α1 through α6; as new integrin α chain proteins were discovered and shown to form heterodimers with β1 integrin, they were assigned numbers; we are now up to α11. αv integrin, first reported to form αvβ3 heterodimers, was originally named for it's ability to bind to vitronectin; αv is included in the β1 family since subsequent to its original naming, it was also shown to form heterodimers with β1.

The β1 family of integrins is present on cells derived from all three tissue types: endodermal, ectodermal and mesenchymal. They are critical for embryonic development. β1 null mouse embryos do not implant (Fassler and Meyer, 1995; Stephens et al., 1995). By creating tissue specific knockout and chimeric mice, β1 integrins have been shown to function in numerous vital processes including organogenesis, hematopoiesis, as well as bone, skin, and hair formation (Potocnik et al., 2000; Raghavan et al., 2000; Bouvard et al., 2001; Globus et al., 2005). Deleting individual β1-integrin family α chains in mice result in milder phenotypes. For example, the α2 knockout mouse appears normal, the α3 knockout mouse has defects in basement membrane assembly in kidney as well as in skin and hair formation; the α4 knockout mouse dies prior to birth due to defects in blood vessel formation (reviewed in Sheppard, 2000).

The β2 integrin family functions primarily in immune cells and has 3 family members (αLβ2, αMβ2, and αXβ2) and its ligands are almost exclusively IgG superfamily members and not extracellular matrix proteins (Mayadas and Cullere, 2005). Patients without β2 integrins on the surfaces of their leukocytes have a condition known as leukocyte adhesion deficiency and are at increased risk of infection since their leukocytes cannot migrate to injury sites efficiently (Lee and Corry, 2004).

αv-Containing integrin heterodimers make up another class of integrins. αv is the only α-chain that can bind to several other integrin β chains. αv can form heterodimers with β3, β5, β6, and β8; including β1, αv can be found in five different integrin heterodimers. All of the αv containing integrin heterodimers recognize the triple amino acid sequence arginine-glycine-aspartic acid (RGD) in their ligands. αvβ3 integrin is important in mediating angiogenesis and therapeutic strategies are in use to block αvβ3 function to reduce the blood flow to certain tumors (Tucker, 2003). The other αv integrins present in the cornea (αvβ5, αvβ6, and αvβ8) mediate transforming growth factor β (TGFβ) activation (Sheppard, 2004).

In addition to these major functional groups of integrins, integrin heterodimers are formed that also have important functions but are difficult to classify. αIIbβ3 is found on platelets and the study of this integrin lead to the discovery of an important family of molecules collectively called the disintegrins which interfere with platelet aggregation and can be used to restore blood flow after heart attack or stroke (Marcinkiewicz, 2005). In addition to αIIbβ3 and αv-family integrins, the α5β1 integrin also binds to it's ligand, fibronectin, via an RGD sequence. αEβ7 is another integrin with unique properties (Strauch et al., 2001; Hadley, 2004). It is expressed by immune cells such as plasma and mast cells and allows these cells to migrate into epithelial tissues by binding to E-cadherin. It plays important roles in immune surveillance, cancer, and allograft rejection after organ transplant.

Finally, α6 which was first described as forming an α6β1 complex, can also partner with the β4 integrin subunit. α6β4 is a component of the hemidesmosomes (Stepp et al., 1990) and is also reported to be a survival factor and therefore to play important roles in carcinogenesis (Tennenbaum et al., 1995; Jones et al., 1998; Mariotti et al., 2001; Chung and Mercurio, 2004; Guo and Giancotti, 2004). Mice lacking either α6 or β4 integrins die at birth; their skin separates at the dermal-epidermal junction due to the lack of hemidesmosomes (Dowling et al., 1996; Georges-Labouesse et al., 1996; van der Neut et al., 1996). All of the integrins discussed previously interact with the actin microfilament component of the cytoskeleton (Fig. 1A). However, unique sequences within the β4 cytoplasmic domain give α6β4 the ability to bind not to actin but to intermediate filament proteins. β4 integrin has a much larger and more complex cytoplasmic segment than any of the other integrins with several distinct functional domains within it that mediate hemidesmosome assembly and regulation of cell survival (Mariotti et al., 2001). Along with type XVII collagen/BPA180, α6β4 is one of two known integral membrane components of the hemidesmosomes (Gipson et al., 1993; Van den Bergh and Giudice, 2003).

Different αβ chain combinations impart distinct ligand binding capabilities to integrin complexes; some αβ heterodimers bind to single ligands whereas others bind to several ligands. Important examples for the cornea are various integrins that mediate adhesion to fibronectins, laminins, and collagens. α1β1 and α2β1 are often referred to as the canonical integrin collagen receptors. The corneal epithelium and stroma express α2β1 as well as α11β1 (Tiger et al., 2001), also a collagen receptor. As integrin αβ heterodimers and new collagens become better characterized, additional collagen binding integrins will be identified some of which are likely to show preferences for distinct collagen types.

Laminin receptors found in the cornea include α3β1 and α6β4. While α1β1 and α2β1 are primarily collagen receptors and α3β1 and α6β4 primarily laminin receptors, all four of these integrins can bind to members of both classes of matrix molecules. These data will no doubt get further refined with improved methods to measure cell adhesion to specific ligands. The fibronectin-binding integrins in the cornea include several distinct αv integrins (αvβ3, αvβ5, αvβ6) and α9β1. Not all

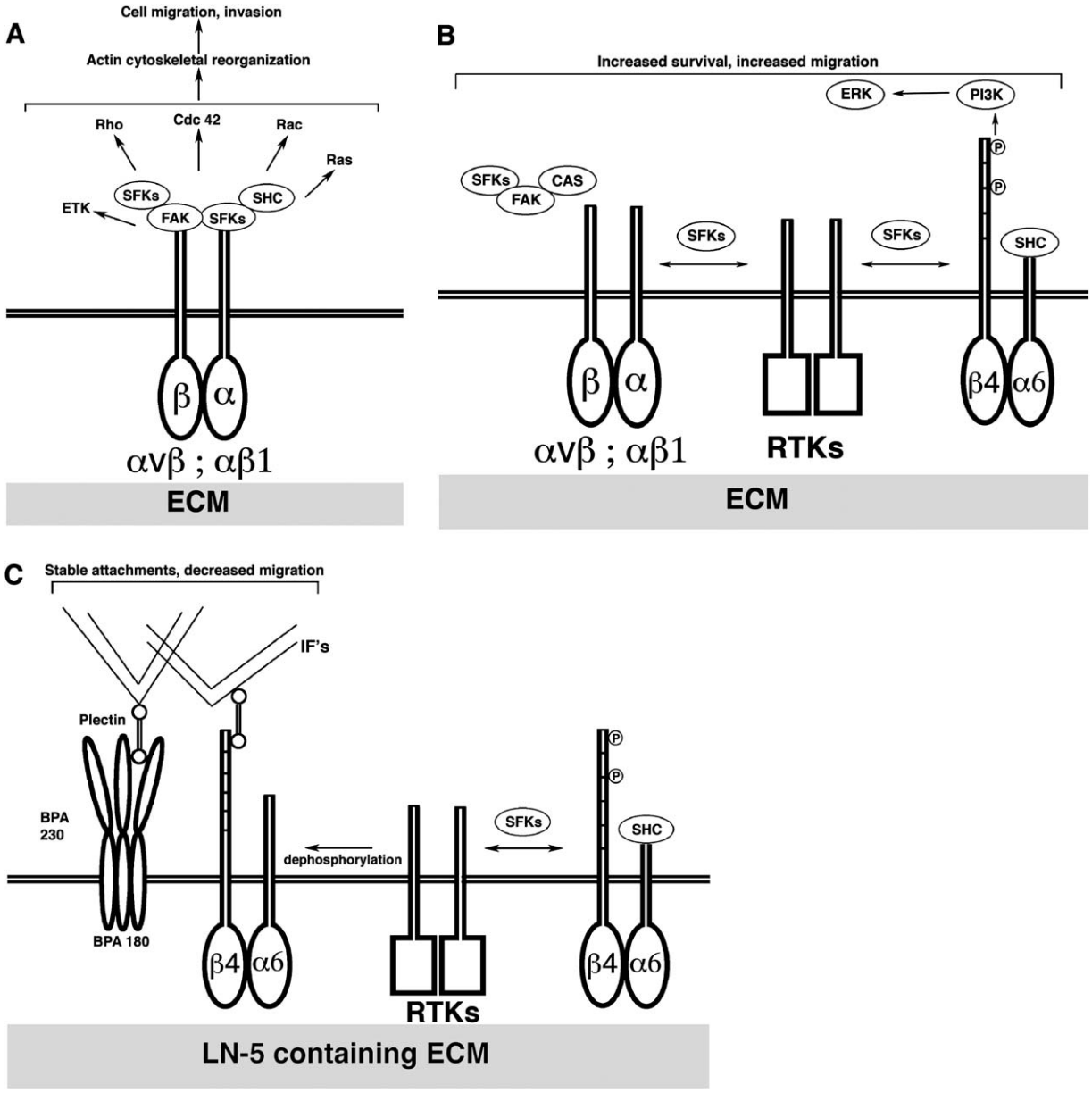

Fig. 1. Schematic representation of integrin functions in the cornea. This image is modified from that of Guo and Giancotti (2004). In the cornea, the possible identities of the αβ1 and αvβ combinations in A and B are listed in Table 1. For epithelial cells, keratocytes/fibroblasts, and endothelial cells, the exact RTKs (receptor tyrosine kinases) and SFKs (Src-family kinases) whose activities are modulated by integrins vary in a tissue specific manner. FAK is focal adhesion kinase, SHC and CAS are adaptor proteins, ECM is extracellular matrix, LN-5 refers to laminin-5 (laminins 332) containing ECM, which is important in hemidesmosome assembly. See text for a more detailed description.

integrins that can bind to fibronectin are expressed in the normal unwounded cornea. Some, such as α9β1 (Stepp and Zhu, 1997) and αvβ6 (Hutcheon et al., 2005), are upregulated upon corneal injury.

While the repertoire of possible integrin heterodimers is huge given all the α (α1–α11, αv, αE, αIIb, αM, αL, αX) and β (β1–β8) chain combinations possible, tissues actually make highly restricted sets of integrin heterodimers. In this review, the different integrins produced by the corneal epithelial cells, the keratocytes, and the corneal endothelial cells will be discussed with special attention to those with clinical relevance. Table 1 lists the corneal integrins identified in the cornea, their CD names if assigned, their localization within the cornea, and reported ligands. Fig. 1A–C shows a schematic representation of the major functions associated with integrins. While some attention will be spent on the different ligands recognized by these integrins and their distribution and functions within corneal tissues, it is necessary to focus here on the integrins themselves.

2. Integrins in the corneal and limbal epithelium

In the corneal epithelium several integrins are expressed in a pattern that is indicative of their function. In human and

Table 1
Integrins reported in the cornea

Family	Subunits present	CD#	Heterodimers reported	Known ligands	Endothelium	Keratocytes in situ	Fibroblasts/ Myofibroblasts in vitro	Epithelium including limbal basal cells	Nerve-associated	Immune cell-associated
β1	α2	CD49b	α2β1	CN, LN		✓	✓	✓		
CD29	α3	CD49c	α3β1	CN, LN, FN		✓	✓	✓	✓	
	α4	CD49d	α4β1	FN-IIIA			✓			
	α5	CD49e	α5β1	CN, LN, FN-RGD			✓			
	α6	CD49f	α6β1	CN, LN, FN		✓	✓	✓	✓	
	α9	CD49i	α9β1	FN-EIIIA, TNC, fOpn, vWF, FBN, FVIII, TG, VEGF-C				✓		
B2	αX	CD11b	αMβ2	ICAMs, FBN						✓
CD18	αM	CD11c	αXβ2	iC3b						✓
αV	β1	CD29	αvβ1	LN, FN via RGD		✓	✓	✓	✓	
CD51	β3	CD61	αvβ3	FN, VN, Fbn, vWF, Tsp via RGD	✓	✓	✓			
	β5	–	αvβ5	CN, LN	✓			✓		
	β6	–	αvβ6	VN, FN via RGD				✓		
	β8	–	αvβ8	LN, FN via RGD					✓	
β4 CD104	α6	CD49f	α6β4	CN, FN, LN-5				✓		

The integrins reported to be present in the various different tissues that make up the cornea are shown. The presence of a check indicates that at least one published report suggests that the integrin is present at the indicated site. See text for citations. For β5, β6, and β8, dashes indicate that no CD numbers have been assigned. Since there have been differences reported in the integrins present in situ in the stroma and in cultured fibroblasts derived from the stroma, those two are considered separately. In cultured corneal epithelial cells derived from the limbus, α5β1 integrin is upregulated and α9β1 is down regulated in culture. The abbreviations used for the various ligands are: CN, collagens; FVIII, Factor VIII; FX, Factor X; Fbn, fibrinogen; FN, fibronectin; FN, EIIIA, fibronectin containing the alternatively spliced EIIIA domain; ICAMs, intercellular adhesion molecules 1–3; iC3b, complement factor 3b; LN, laminins; LN-5, laminin-5; fOpn, a fragment of osteopontin; RGD, the three amino acid sequence (arginine-glycine-aspartic acid) recognized by some integrins; TNC, tenascin-C; TG, transglutaminase; Tsp, thrombospondin; VN, vitronectin; vWF, von Willebrand factor.

mouse central cornea, there is α2β1, α3β1, αvβ5, and α6β4 all of which have a polarized localization within the epithelium (Stepp et al., 1990, 1993; Grushkin-Lerner and Trinkaus-Randal, 1991; Tervo et al., 1991; Murakami et al., 1992; Paallysaho et al., 1992; Latvala et al., 1996). They are expressed most intensely in the basal cells with expression progressively lost in the more apical layers. The most intense staining for integrins is found where the basal aspect of the basal cells comes into contact with the basement membrane. Here integrins mediate attachment to matrix proteins in the basement membrane via both focal adhesions that are actin-based and hemidesmosomes that are intermediate filament based. Integrins can also be found in intracellular compartments and at lateral and apical membranes of the basal cells. From experiments performed on cells derived from rabbit (Nakagawa et al., 1990), rat (Stepp et al., 1993), and human (Filenius et al., 2001, 2003; Li et al., 2005a,b) corneas, we know that integrins mediate adhesion of corneal epithelial cells to collagens, fibronectins, laminins, and vitronectin. While adhesion to some of these matrix molecules is inhibited by RGD peptides, not all adhesion is RGD-sensitive suggesting that adhesion is a cooperative event involving multiple different integrin heterodimers.

The basal cell layer is the least differentiated, most proliferative, and expresses the most integrin. When integrin expression decreases, cells become both less proliferative and less adhesive to the underlying basement membrane. When adherent cells are prevented from adhering to matrix, they undergo a form of apoptosis called anoikis (Frisch and Francis, 1994). In the past decade, anoikis has been studied in detail (Grossmann, 2002; Valentijn et al., 2004); it results from disruption of the interplay that normally occurs between integrins and growth factor receptor tyrosine kinases (RTKs) such as those that recognize and bind PDGF and EGF. Integrin engagement reinforces signaling from RTKs (Fig. 1B). Without integrins to sustain RTK signaling, these kinases are inactivated or internalized making it impossible for added growth factors to be sensed by the cell. The reduction in integrin expression that occurs as corneal epithelial basal cells leave the basal compartment not only assures that those cells will divide less frequently, it also makes the apical aspect of the epithelium less adhesive for viruses and bacteria that might exploit integrins ability to bind RGD-containing ligands as a means to get into and infect cells (Goldman and Wilson, 1995; Goldman et al., 1996).

The corneal epithelium, due to its accessibility, has been studied for many years as a model tissue for investigating the molecular changes that occur during wound healing. In the mid-1980s reports showed that application of topical fibronectin to the cornea after wounding could help the healing of corneal wounds in rabbits (Nishida et al., 1983, 1984). Additional studies confirmed these results (Watanabe et al., 1987) and studies in patients with corneal ulcers began (Phan et al., 1987). While early studies need to be reinterpreted in light of data showing that growth factors such as EGF often co-purify with fibronectin and together enhance re-epithelialization (Nishida and Tanaka, 1996), they excited the interest

of many groups hoping to identify the integrins that were expressed in the cornea during wound healing.

After demonstrating that β1-family integrins were synthesized by rabbit corneal epithelial cells in culture (Trinkaus-Randall et al., 1990), Grushkin-Lerner and Trinkaus-Randal (1991) went on to show that there were changes in expression of integrins during migration after debridement and keratectomy wounds. Following our demonstration that α6β4 was a hemidesmosomal component (Stepp et al., 1990), interest in hemidesmosomal disassembly and reassembly grew. With improved antibodies and in vivo experiments, were we able to demonstrate the upregulation of α6β4 integrin in suprabasal cell layers in the rat during re-epithelialization in vivo but not in vitro in organ cultures (Stepp et al., 1993, 1996). Others have looked at changes in α6β4 integrin during re-epithelialization in rabbits using α6 specific antibodies and shown similar results (Paallysaho et al., 1992; Latvala et al., 1996). The fact that α6β4 expression increases when the hemidesmosomes are largely disassembled during re-epithelialization reaffirms roles for this integrin distinct from those associated with hemidesmosome assembly. In the past several years, these roles have been studied in much more detail in the skin, in tumors, and in the cornea. Data are summarized in the cartoon presented in Fig. 1B and C.

α6β4 is known to be a survival factor for epithelial cells (Chung and Mercurio, 2004; Mercurio et al., 2004); increased expression and function is associated with invasion and metastasis of various epithelial derived tumors. It interacts with receptor tyrosine kinases such as the EGF and PDGF receptors to sustain growth factor receptor signals. Along with α3β1, it regulates cell cycle progression in epithelial cells. The αv-family integrins including αvβ1 (Munger et al., 1998), αvβ5, αvβ6, and αvβ8 (Sheppard, 2004) mediate activation of latent TGFβ and thus influence signaling from the receptor tyrosine kinases that bind TGFβ family members (Munger et al., 1998). TGFβ has important affects on corneal epithelial cells in culture; it can increase extracellular matrix expression, matrix metalloproteinase expression, and promote terminal differentiation of epithelial cells (Wenner and Yan, 2003; Kim et al., 2004). Also, the TGFβ secreted by epithelial cells, if active, can act on the underlying corneal fibroblasts. It appears that α6β4 mediates epithelial cell survival and cell adhesion via hemidesmosomes and, αv-integrins can, by regulating TGFβ activation, regulate epithelial cell:matrix interactions and differentiation.

There were initially confusing results regarding expression of α5β1 in corneal epithelial cells in vivo (Trinkaus-Randall et al., 1990; Stepp et al., 1993). α5β1 was detected when studies were performed on cultured corneal epithelial cells (Nishida et al., 1992; Maldonado and Furcht, 1995). Also, initially, there were few high quality antibodies available for non-human studies. Now it is accepted that normal epidermis and corneal epithelium lacks α5β1 and αvβ3.

Integrins mediate cell migration both by transmitting forces from the matrix to the cytoskeleton and by regulating changes in cytoskeletal organization. This allows for the rapid shape changes needed during cell migration (Sheetz et al., 1998; Li et al., 2005a,b). Cell migration involves numerous Src family kinases and focal adhesion kinase (Fig. 1A). Integrin engagement and clustering affects the activity of these molecules and leads to the development of cell polarity during migration establishing a leading edge and a trailing edge. As stationary corneal epithelial cells begin to migrate, activated kinases phosphorylate the β4 cytoplasmic domain, which induces hemidesmosome disassembly (Mariotti et al., 2001). Small focal-adhesion-like plaques form beneath cells at the leading edge; as cells crawl forward, focal-type adhesions accumulate at the trailing edge. The detachment of the rear of the cell and not extension of the leading edge, at least in fibroblasts, is believed to limit the rate of migration (Cox and Huttenlocher, 1998; Kirfel et al., 2004). It is achieved both by the action of matrix metalloproteinases secreted at this site and by cells leaving behind patches of cell membrane, referred to as footprints or migration tracts.

In epidermal keratinocytes, α3β1 has been studied most extensively during cell migration (Goldfinger et al., 1999; Giannelli et al., 2001). It regulates the expression and secretion of matrix metalloproteinases that assist in regulating cell movement. Migrating keratinocytes secrete intact laminin-5, comprised of three chains called α3β3γ2, which accumulates beneath the cells. Over time, as the cells migrate, the laminin α3 chains are cleaved by the action of metalloproteinases. Both α3β1 and α6β4 bind to preferentially to processed forms of the laminin-5 α3 chain. The heparan sulfate proteoglycan syndecan-1 binds preferentially to unprocessed forms of the laminin-5 (Okamoto et al., 2003). Quiescent epithelial cells with intact hemidesmosomes have only cleaved laminin-5 beneath them so that α3β1 and α6β4 adhesions are in their most stable conformation. Syndecan-1 is never localized beneath the basal aspect of the epithelial cell but is present between the cells. During migration, unprocessed laminin-5 is secreted and accumulates on top of the processed laminin-5 so stable adhesions via α3β1 and α6β4 are not possible. Syndecan-1 is also upregulated during migration and may play a role in modulating integrin affinity for the matrix. Because of the newly secreted unprocessed laminins and the presence of syndecan-1 beneath cells, the adhesions that form are more favorable for cell migration.

αv-Family integrins αvβ5 and αvβ6 no doubt also play important roles in mediating cell migration (Varadarajulu et al., 2005). Recent studies in epithelial to mesenchymal transformation and the role it plays in metastasis show αvβ6 as an important regulator of E-cadherin expression (Bates and Mercurio, 2005); loss of αvβ6 leads to a loss of adherens junctions and epithelial cell identity. Perhaps expression of αvβ6 during re-epithelialization after specific types of corneal wounds but not others (Hutcheon et al., 2005) helps to maintain E-cadherin expression and maintain sheet integrity.

α9 integrin has been studied in the cornea during cell migration in the mouse. Increased expression is observed during re-epithelialization at later time points after both small and large debridement wounds (Stepp and Zhu, 1997; Sta Iglesia et al., 2000; Pal-Ghosh et al., 2004). α9 integrin recognizes numerous ligands including the alternatively spliced domain of fibronectin called EIIIA. One concern of those who study

the role of fibronectin in wound healing is that fibronectin is such an abundant serum protein that it was hard to separate out the affects of fibronectin that come from serum from those from the fibronectin made by cells. Cai et al. (1993) resolved that issue by using in situ RT–PCR to study production of fibronectin mRNAs. By looking at changes in fibronectin mRNA expression within corneal wounds as they healed, they were able to show that migrating corneal epithelial cells altered their expression of mRNAs for various alternatively spliced isoforms of fibronectin including the EIIIA domain. It took 11 years to confirm that the EIIIA domain was present in the fibronectin protein deposited in the provisional matrix during wound healing in the rat cornea (Havrlikova et al., 2004) and the skin (Singh et al., 2004). While we do not know for sure if α9 integrin is functioning during re-epithelialization by binding to FN-EIIIA, the timing of the changes in mRNAs and protein for both α9 integrin and FN-EIIIA make this seem likely. The regulation of α9 integrin and alternatively spliced fibronectin isoforms is among the best characterized of the integrin:ligand interactions that occur during wound healing. Studies have evaluated both receptor and ligand mRNAs by in situ hybridization and co-distribution of the proteins has been reported in healing rat skin. In culture, when expression is induced, α9 integrin has been shown to mediate cell migration by regulating association of the protein paxillin with the cytoskeleton (Young et al., 2001). It is likely that α9β1 integrin is involved in sustaining migration on FN-EIIIA once it has been initiated rather than in mediating the transition of cells from a quiescent to a migratory state; however this remains to be proven.

Finally, another area that needs to be studied more involves α6β4 and whether or not it is actively involved in mediating the mechanics of cell migration. The fact that α6β4 mediates intermediate filament insertion at the cell membrane rather than actin microfilament insertion has lead to the assumption that it plays primarily a passive role in allowing cells to migrate: by remaining phosphorylated during migration, it prevents premature reassembly of hemidesmosomes. However, numerous studies by Mercurio et al. (2004) suggest a much more active role for α6β4 in cell migration and metastasis. It is not clear at present exactly what role(s) it may play but this remains an exciting area of research.

There are numerous studies showing that diseases of the cornea involve changes in epithelial integrin expression and/or localization. These conditions include bullous keratopathy, diabetes, keratoconus, recurrent erosion, and stem cell deficiency; data are summarized in Table 2. In addition, there are changes in integrin localization associated with aging that may contribute to difficulties in healing. In patients with bullous keratopathy, increased levels of α2β1 and α3β1 have been observed (Vorkauf et al., 1995). In addition, elevated levels of α8β1, α9β1, and αvβ6 integrins were also seen in corneas of patients with bullous keratopathy (Ljubimov et al., 2001); all of these integrins share the ability to bind to tenascin, a protein that had been observed to accumulate in the stromas of patients with disease (Ljubimov et al., 1998). In contrast, another study has shown that α6β4 integrin was decreased in corneas of patients with bullous keratopathy (Spirin et al., 1999). Diabetic patients show delayed wound healing and their corneas have been evaluated for changes in integrin expression as well. Data show decreased expression of α3β1 and increased expression of matrix metalloproteinase -10 (Saghizadeh et al., 2001, 2005; Kabosova et al., 2003). These results are interesting in that α3β1 expression is in part responsible for regulating matrix metalloproteinase expression in cultured keratinocytes (DiPersio et al., 1997).

Keratoconus corneas are thinner and more steeply curved than normal corneas. Cheng et al. (2001) found that there was reduced expression of type XII collagen in keratoconus corneas but similar distributions of β1 and β4 integrins. Ebihara et al. (2001) reported suprabasal expression of α6β4 whereas both Vorkauf et al. (1995) and Tuori et al. (1997)

Table 2
Changes reported in corneal epithelial integrins

Condition	Integrins altered	Model	References
Debridement wound	↑α9β1	Rabbit; mouse; rat	Grushkin-Lerner and Trinkaus-Randal, 1991; Latvala et al., 1996; Stepp et al., 1996; Stepp and Zhu, 1997
	↑α6β4		
Keratectomy wound	↑αvβ6	Rabbit; rat	Grushkin-Lerner and Trinkaus-Randal, 1991; Paallysaho et al., 1992; Murakami et al., 1992; Hutcheon et al., 2005
	↑α6β4		
	↑α9β1		
Recurrent erosion	↓α9β1	Mouse	Pal-Ghosh et al., 2004
Stem cell deficiency	↑α9β1	Mouse	Pal-Ghosh et al., 2004
Bullous keratopathy	↑α2β1	Human	Vorkauf et al., 1995
	↑α3β1		
	↑αvβ6	Human	Ljubimov et al., 1998, 2001
	↑α8β1		
	↑α9β1		
Diabetes	↓α3β1	Human	Saghizadeh et al., 2001; Kabosova et al., 2003
Keratoconus	Altered α6β4	Human	Vorkauf et al., 1995; Tuori et al., 1997; Ebihara et al., 2001; Cheng et al., 2001
	↓α3β1		
Aging	Altered α6β4	Human	Trinkaus-Randall et al., 1993

Several reports indicate that there are changes in integrin expression and/or localization in various different disease states and with aging. The condition studied, the integrin heterodimer whose expression is altered, whether the integrin was reported to be up or down regulated, whether the report refers to humans or animal models, and the relevant citations are indicated. For keratoconus and aging, the changes reported are more complex (see text).

report reduced expression of β4 integrin in keratoconus. While it is possible that the different results reported by these groups were due to differences in antibodies or methods of fixation or staining, it is also possible that these results reflect variations among keratoconus corneas since the disease is genetically variable.

In the aging cornea, numerous changes occur that put the cornea at risk. Corneal sensitivity decreases, there is reduced resistance to infections, and increased epithelial permeability to fluorescein (Faragher et al., 1997). A study of aging human corneas showed that α6β4 localization at the basement membrane zone was discontinuous (Trinkaus-Randall et al., 1993). Given the newly appreciated roles of αv-family integrins in mediating adenoviral infections coupled with the reduced resistance to infection seen in aging eyes, it would be useful to look at additional integrins besides α6β4.

Numerous corneal dystrophies are being characterized at the genetic level; for some, it is likely that changes in integrin expression or function will be seen when evaluated more closely. For example, several dystrophies (gelatinous drop-like, lattice, Avellino, Reis-Bucklers) are all linked to mutations in the gene TGFB1/BIGH3, which encodes the protein called transforming growth factor β-induced protein-1p (TGFB1p). Recent studies have shown that TGFB1p binds to collagen VI (Andersen et al., 2004) and can also interact with several integrins that are expressed in the cornea including α3β1, αvβ5, and α6β4 (Bae et al., 2002; Kim et al., 2002). Peptides derived from TGFB1p are antiangiogenic (Nam et al., 2003). It will be interesting to see whether corneas derived from patients with mutations in this gene have altered integrin expression and localization.

The causes of recurrent erosion and stem cell deficiency conditions in the cornea are poorly understood. In the Balb/c mouse, we recently reported the characterization of a model for the study of recurrent erosions (Pal-Ghosh et al., 2004). When small or large manual debridement wounds are made to the surface of the mouse cornea, wounds reseal within days but not permanently. When times up to 8 weeks after wounding were evaluated, we saw discrete sites where patches of epithelial cells had spontaneously eroded. These were not sterile ulcers since the erosion sites were closed prior to reopening. After large wounds, these erosions are accompanied by goblet cell invasion of the central cornea, a condition often called Corneal Epithelial Stem Cell Deficiency; after smaller wounds, we never see progression to stem cell deficiency. Interestingly, stem cell deficiency was accompanied by a loss of α9 integrin at the sites where goblet cells crossed over the limbus onto the cornea. The presence of α9 integrin within the epithelial cells of the limbus appears to protect the surrounding corneal tissue from invasion by goblet cells. We do not yet know if stem cell deficiency in humans is also associated with decreased α9 integrin production; limited availability of antibodies for staining tissues for α9 integrin has slowed research. Our laboratory is currently developing monoclonal antibodies against α9 integrin to address this issue.

It is clear that α3β1 and α6β4 play roles in cell survival. It is not clear, however, the roles they may play in maintaining the corneal epithelial stem cell population at the limbus. Several studies suggest correlations between integrin expression and the maintenance of the adult stem cell population at the limbus (Pajoohesh-Ganji and Stepp, 2005; Schlotzer-Schrehardt and Kruse, 2005). We performed whole mount studies on mouse corneas that had been subjected to BrdU labeling as neonates (Pajoohesh-Ganji et al., 2005). We correlated expression of BrdU with that of β1, β4, and α9 integrins. Data from those studies suggest that the slower cycling label retaining cells at the limbus express higher levels of β1 and β4 than do the surrounding cells at the limbus. While its expression is restricted in adult corneas to a subset of limbal basal cells, α9 integrin is not a stem cell marker but rather is expressed on rapidly cycling transiently amplifying cells. Other studies suggest similar roles for β1 and β4 integrins in epidermal stem cells (Jones and Watt, 1993; Tani et al., 2000). Knockout mice with skin targeted deletions of β1 integrin have defects in hair follicle development that are due, in part, to the inability of the epidermal stem cells to divide asymmetrically in the absence of β1 integrins (Lechler and Fuchs, 2005). In developing skin, α3β1 is known to organize the assembly of the basement membrane (DiPersio et al., 1997). Without the proper cues from the basement membrane, asymmetric cell division was absent in the embryonic skin of β1 null mice; β4 null embryonic skin was able to proceed past the first asymmetric cell division and maintain its stem cell population longer than the skin of β1 null mice (Lechler and Fuchs, 2005).

Interest in stem cells has grown and with it, efforts to grow and maintain corneal epithelial stem-like cells in culture. Human corneal epithelial cells have been grown in culture for many years (Ebato et al., 1987, 1988; Haskjold and Nicolaissen, 1988) and scientists were quick to see that cells obtained from donor limbal rims grew better in culture than did cells obtained from the central cornea. When placed in culture, human corneal epithelial cells upregulate α5β1 integrin and show adhesion and spreading properties distinct from those seen in freshly isolated cells (Nakagawa et al., 1990). The fact that human limbal cells grew better than central corneal cells in culture along with observations on label retaining cells in situ (Lehrer et al., 1998), lead to the limbal stem cell hypothesis (reviewed in Daniels et al., 2001). This hypothesis states that the stem cells for the central cornea are found at the limbus and that as daughter cells divide and move towards the center of the cornea, they become increasingly restricted in their proliferative potential (reviewed in Stepp and Zieske, 2005).

It has long been hoped that cultured human corneal epithelial cells could be used to treat patients with stem cell deficiency; several studies have been published and the results so far are promising (Grueterich et al., 2002; Espana et al., 2003; Koizumi and Kinoshita, 2003). One group has directed their efforts towards using a selection of cultured human corneal epithelial cells based, at least in part, on integrin function to improve the chances for success for corneal epithelial stem cell transplants (Li et al., 2005a,b). The well characterized roles of integrins including α3β1 and α6β4 to function as survival factors makes them good candidates for proteins that are important in maintaining the stem cell population of the

cornea. Understanding ways to manipulate these cells and maintain them in culture is key to improving the quality of life for patients with corneal epithelial stem cell deficiencies.

3. Keratocyte integrins

The fibroblasts that are found in the corneal stroma have been called corneal keratocytes; they synthesize and maintain the bulk of the collagen and proteoglycans that make up the corneal stroma. Their ability to organize the corneal stroma to produce a clear matrix that does not distort incoming light has intrigued researchers for years and remains an important area for ongoing research (Muller et al., 2004). The keratocytes in the corneal stroma are thought to be relatively quiescent in normal adult corneas. They form a network of cells and have been shown both in vivo and in vitro to be communicating with one another via gap junctions (Jester et al., 1994; Petridou and Masur, 1996; Spanakis et al., 1998). It is likely that the entire corneal stroma is functionally interconnected via keratocyte gap junctions.

The keratocytes undergo a remarkable transformation upon activation (Jester et al., 1999; Masur et al., 1999; Wilson et al., 2003). They convert into cells known as myofibroblasts which, together with adjacent keratocytes, act to contract the wound bed, allowing for more rapid wound closure by the epithelial cells. After wounds are healed, myofibroblasts either undergo apoptosis or revert back into quiescent keratocytes (Mohan et al., 2003). Depending upon the nature of the wound, scars can form at sites of myofibroblast activation and can interfere with vision depending upon their location in the visual field. With the advent of various surgical methods to manipulate the curvature of the cornea (PRK, LASIK, LASEK), interest in the roles played by keratocyte activation in corneal scaring and haze has increased.

When quiescent keratocytes are placed in culture they get activated in response to serum and other growth factors to proliferate. Their morphology changes from a dendritic to a more well-spread phenotype; serum-activated keratocytes in culture have been called fibroblasts. Over time, a percentage of the fibroblasts will convert into myofibroblasts, which are contractile and express smooth muscle actin. Using cultured stromal fibroblasts obtained from rabbits, Masur et al. (1993, 1999) looked at integrin expression biochemically. Cultured fibroblasts were shown to express αvβ3, α4β1, α5β1, α6β1, and α3β1. In their experiments they did not look at α2β1 or at other αv-containing integrin heterodimers. When compared to keratocytes derived directly from rabbit corneal stroma prior to cell culture, fibroblasts made α5β1. A study by Jester et al. (1994) confirmed that α5 integrin was not expressed by cat keratocytes in situ but α5 and additional β1-family integrins were induced upon cell culture.

Conversion of quiescent keratocytes to myofibroblasts is regulated by autocrine TGFβ signaling (Petridou et al., 2000) and can be induced by the addition of TGFβ to corneal fibroblasts (Jester et al., 1996; Masur et al., 1999). Fibroblast to myofibroblast conversion can be inhibited by RGD peptides, inhibitors of PDGF function (Jester et al., 2002), or by inhibition of Smad 2/3 signaling (You and Kruse, 2002). The conversion is also reversible: myofibroblasts can convert back to fibroblasts if incubated in the presence of FGF (Maltseva et al., 2001). Thus, conversion from fibroblast to myofibroblast results in changes in integrin localization and function and the signal transduction events that lead to the formation of myofibroblasts from fibroblasts involve PDGF, TGFβ, and the RGD-sensitive integrins (αv family members and/or α5β1).

Imoto et al. (2003) were interested in the effects of exogenous RGD peptides on several different cell types obtained from the human eye including corneal fibroblasts. They assessed integrin expression in cultured human corneal fibroblasts by FACS analysis and found that α2 subunit containing integrins were the dominant integrins expressed but that α3, α5, and αv could also be detected. α2β1 integrin:collagen interaction is generally considered to not involve RGD sequences. One important function identified for α2β1 integrin in vascular smooth muscle cells is its ability to mediate contraction of collagen gels (Lee et al., 2005). Thus, even though conversion of keratocytes to myofibroblasts involves the RGD sensitive integrins, collagen binding integrins that are not RGD sensitive like α2β1 and α11β1 likely mediate wound contraction by myofibroblasts.

The assembly and maintenance of the collagen: proteoglycan matrix by stromal keratocytes is likely regulated by integrins though data in the cornea is insufficient. Velling et al. (2002) have shown that polymerization of type I and III collagen is dependent on fibronectin, and is enhanced by α2β1 and α11β1 integrins in embryonic mouse fibroblasts. The ability of α5β1 to regulate fibronectin matrix assembly has been studied in detail (Wennerberg et al., 1996; Lohikangas et al., 2001; Mao and Schwarzbauer, 2005). α3β1 integrin regulates basement membrane assembly by mediating the ability of cells to interact with and organize laminin (Dipersio et al., 1997; deHart et al., 2003).

What emerges from these data is the concept of a corneal keratocyte with elongated processes that forms an anastomosing network within the corneal stroma. Each fibroblast forms connections with numerous other keratocytes with which they communicate directly via gap junctions. A study of corneal nerves and their role in mediating keratoconus revealed that vimentin-positive, α3β1 integrin negative, keratocytes were often intimately associated with the corneal nerves, sometimes wrapping around them prior to their penetration of Bowman's layer (Brookes et al., 2003). In human studies, it wasn't clear whether the keratocytes were contacting the axonal membrane or Schwann cell membrane directly. Interestingly, Schwann cells were positive for α3β1 integrin and they also produce a collagen IV-rich lamina that wraps around each axon (Bee et al., 1988) raising the possibility that additional β1 family integrins are present. A close consideration of the work of Brookes et al. (2003) makes it clear that earlier studies of keratocyte integrins in situ could well have confused Schwann cell β1 family integrins for keratocyte integrins.

To summarize, in extracts obtained from normal corneal stroma, quiescent keratocytes are reported to express the

following integrins: αvβ3 as well as α2β1, α3β1, and α6β1; one group has also shown α4β1. Data consistently show no α5β1 in the unwounded corneal stroma. Subsequent studies performed using immunocytochemistry and immunofluorescence microscopy have shown that at least a portion of the integrins present in these extracts could be derived from Schwann cells and not from keratocytes. Placing keratocytes in culture in the presence of serum yields corneal fibroblasts, which produce the same integrins as those found in situ plus α4β1 and α5β1. Adding TGFβ induces the fibroblasts to convert to myofibroblasts and alters cell shape and morphology. This conversion is integrin dependent; since it can be blocked by RGD peptides, α5β1 and/or αvβ3 integrins are likely to be involved. Integrins are likely to regulate not only contraction of wounds by myofibroblasts, but also the ability of keratocytes and myofibroblasts to assemble and maintain their collagen and proteoglycan-rich matrix and, therefore are critical in resolving corneal haze and scaring after injury.

By using cell culture models, our understanding of the biochemical and signaling events that regulate corneal keratocytes and myofibroblasts has grown considerably over the past several years. However, at a molecular level, the important events that occur in situ within the corneal stroma after wounding in vivo are still not clear (Netto et al., 2005). It is not known whether keratocyte conversion to myofibroblasts in vivo is accompanied by changes in integrin expression and function and whether or not it is reversible. It is also unclear whether corneal haze is the product of activated stromal keratocytes, activated immune cells, or both. Only recently have we begun to recognize that resident immune cells, Schwann cells, and nerve-derived peptides (Sassani et al., 2003) likely play roles in the wound response of the cornea. In addition, the roles that may be played by recruited bone marrow-derived immune-like cells in the wound response of the corneal stroma are only beginning to be investigated (Nakamura et al., 2005; Sosnova et al., 2005). Now that the important players are all identified, progress in our understanding of wound healing in the corneal stroma in vivo should be rapid.

4. Evidence for integrins in the corneal endothelium

Evidence for integrins in the corneal endothelium is mostly indirect rather than direct. Bovine corneal endothelial cells have been grown in culture for over 27 years and matrix derived from them has served as an important substrate for the growth of numerous other cell types (Gospodarowicz, 1979). In fact, the use of bovine corneal endothelial cell cultures and matrix derived from them lead directly to advances in our understanding of extracellular matrix proteins like fibronectin and various collagens and indirectly to the discovery of the fibroblast growth factors (Gospodarowicz et al., 1978). In 1979, bovine corneal endothelial cells were first used in in vivo transplantation studies in rabbit corneas (Gospodarowicz et al., 1979). In the 26 years since publication of studies using bovine corneal endothelial cells, we have learned a lot about the human corneal endothelium but routine transplantation of human corneal endothelial cells is still not a reality (Engelmann et al., 2004). Bovine corneal endothelial cells grow readily in culture but normal human corneal endothelial cells were found to be difficult to grow and only recently with improved understanding of growth factors and more defined culture media have methods become available for their maintenance and growth in culture (Zhu and Joyce, 2004).

In intact corneas, Descemet's membrane itself is a barrier to a better understanding of integrin expression by corneal endothelial cells. It's rich extracellular matrix composition and ability to bind and sequester growth factors also give it the unfortunate affect of promoting the non-specific binding of antibodies. While studying the epithelial basement membrane zone of diabetic human corneas, Ljubimov et al. (1998) state that the following integrins are present in endothelial cells at Descemet's membrane: α2β1, α3β1, α5β1, and α6β1 and that there were no differences between the integrins present at Descemet's in normal verses diabetic corneas. However, the endothelial cell data were not presented in that study whose main focus was differences in epithelial integrins. Joyce et al. (1998) were assessing the nature of the mitotic inhibition observed in the post-natal developing rat corneal endothelium and they reported that αvβ3 integrin was expressed by rat corneal endothelial cells early in post-natal corneal maturation. It went from being localized at both lateral and basal sites in neonates between 1 and 7 days of age to being present exclusively at the basal aspect of the corneal endothelial cells between 14 and 21 days. Those studies utilized a β3 integrin specific antibody and cannot rule out the presence of additional αv-containing integrins at Descemet's. Studying the adhesion properties of bovine corneal endothelial cells to various different collagens implicated adhesion via integrins. Data showed that fibronectin and RGD peptides could partially block cell adhesion to collagen IV (Rixen et al., 1989). These results implicate αv-family integrins on the surface of corneal endothelial cells as mediators of cell adhesion to collagen since the other collagen receptors, α1β1 and α2β1, do not function by binding through RGD sequences.

One problem for corneal researchers has been that it is difficult to efficiently transfect corneal cells with DNA. With the discovery that adenoviral-mediated gene transfer occurs more efficiently in cells expressing αv-family integrins (Goldman and Wilson, 1995; Goldman et al., 1996), interest in expression of these integrins was increased in the eye community. A recent study using integrin targeted vectors claims to have achieved 100% corneal endothelial cell transfection efficiency (Collins and Fabre, 2004). Such studies confirm indirectly that corneal endothelial cells express integrins that bind ligands via RGD sequences, namely αv-family integrins including αvβ3, and/or α5β1. Rayner et al. (1998) became intrigued with the idea of using adenoviral-mediated gene transfer in the cornea and they studied in detail the expression of the αv subunit as well as β3 and β5 integrin subunits in human corneas. They found that the endothelial cells of all of the eyes studied expressed αv integrin and that it was present not as an αvβ3 heterodimer but coupled with β5. In addition, they found αvβ5 in the corneal epithelial basal cells of 7 of 9 eyes evaluated.

From studies of the matrix produced by corneal endothelial cells and the affects of mutations in some of these genes on Descemet's membrane formation, we know that collagen VIII is an important mediator of corneal endothelial cell attachment and migration (Biswas et al., 2001). When studied in detail in another cell type, smooth muscle cells, adhesion and migration of cells on type VIII collagen was dependent on two β1 family integrins, α1β1 and α2β1 (Hou et al., 2000). While published data support the presence of αvβ3 and αvβ5 on rat and human corneal endothelial cells respectively, it is likely that additional β1-family integrins will be found expressed by these cells. Given the importance of the endothelium to the overall health of the cornea and our ability to exploit integrin function to allow for gene transfection, it is surprising that more is not known about expression of integrins by corneal endothelial cells.

5. Summary

The cornea has been a frequent model used for the study of development and wound healing in birds and mammals. Studies of the stroma have lead to the characterization of collagen and proteoglycan assembly. Despite years of study of the cornea and its matrix, at the molecular level, we still need to know much more about how its cells interact with their matrix to maintain its clarity in health and disease. It has been just over 20 years since β1 integrin was first cloned in the chick (reviewed in Hynes, 2004). Since then, we have learned a lot about integrins in the cornea but there are still many questions to be addressed.

In the epithelium, we are just beginning to learn the adhesion properties of the corneal epithelial stem cells. While we can routinely grow human corneal epithelial cells, we are at a loss to develop routine culture methods for mouse corneal epithelial cells which limits our ability to use cells from genetically altered mice to study signal transduction. Altered integrin expression is seen in several corneal disease states but we do not yet know if this is a cause of disease or an effect. Aging and diabetic corneas show reduced ability to heal epithelial wounds and the roles integrins play have yet to be fully appreciated. In the stroma, we need to study the roles played in wound healing and disease by all of the cell types present including the keratocytes, immune cells, and Schwann cells. Nerve axons release neurotrophic factors in the stroma that can affect the keratocytes and the overlying epithelial cells. Using cell culture models, we have advanced our understanding of the fibroblast to myofibroblast conversion and the roles played by integrins and growth factors. While in vivo studies are more complicated to undertake, by using newly available markers for the various cell types present combined with advances in imaging, rapid progress in understanding how corneas respond to injury will help us better treat those with injuries and corneal dystrophies affecting the stroma.

Bovine corneal endothelial cells were the first corneal cells to be cultured successfully and their culture advanced the field of cell biology by leading to the isolation and characterization of various matrix molecules and growth factors. Once it was clear that human corneal endothelial cells were more difficult to manipulate in culture, progress in understanding the molecular properties of the corneal endothelium slowed dramatically. We know less about corneal endothelial integrins than we do about integrins in the other two corneal tissue layers. Integrins impact cell adhesion, matrix assembly, and cell survival; they are implicated as stem cell markers. The loss of endothelial cells as corneas age has been studied as a cell proliferation defect; however, it could also be caused by changes in integrin function in corneal endothelial cells. The corneal endothelial cells alone are vital for a successful corneal transplant and defects in their ability to provide nourishment from the anterior aqueous humor as they age impacts the viability of the stromal keratocytes and corneal epithelial cells. Further analysis of corneal endothelial integrins and their alterations in corneal disease and with aging is needed to provide important new insight into how the cornea remains healthy.

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