ROLE OF BASEMENT MEMBRANE AND EXTRACELLULAR MATRIX PROTEINS IN THE ADHESION AND SPREADING OF IMMORTALIZED HUMAN CORNEAL EPITHELIAL CELLS

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CONTENTS

1. ORIGINAL PUBLICATIONS (Sissi Katz, neé Hasenson)

This thesis is based on the following articles, which are referred to in the text by their Roman numerals (I-IV).

I Sissi Filenius (Katz), Marketta Hormia, Jan Rissanen, Robert E Burgeson, Yashihiko Yamada, Kaoru Araki-Sasaki, Masatsugu Nakamura, Ismo Virtanen and Timo Tervo. Laminin synthesis and the adhesion characteristics of immortalized human corneal epithelial cells to laminin isoforms. Exp Eye Res 72, 93-103, 2001

II Sissi Filenius (Katz), Timo Tervo and Ismo Virtanen. Production of fibronectin and tenascin isoforms and their role in the adhesion of human immortalized corneal epithelial cells. IOVS 44, 3317-3325, 2003
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III Sissi Hasenson (Katz), Marko Määttä, Patricia Rousselle, Yamato Kikkawa, Jeffrey H Miner, Timo Tervo and Ismo Virtanen. The immortalized human corneal epithelial cells adhere to laminin-10 by using Lutheran glycoproteins and integrin α3β1. Exp Eye Res 81, 415-421, 2005

IV Sissi Katz, Mika Hukkanen, Kari Lounatmaa, Patricia Rousselle, Timo Tervo and Ismo Virtanen. Cooperation of isoforms of laminin-332 and tenascin-C^L during early adhesion and spreading of immortalized human corneal epithelial cells. Exp Eye Res 83, 1412-1422, 2006

2. ABBREVIATIONS

BM	basement membrane
BSA	bovine serum albumin
CLSM	confocal laser scanning microscopy
DAB	diaminobenzidine
DEPC	diethyl pyrocarbonate
DIG	digoxigenin
DOC	Na-deoxycholate
ECM	extracellular matrix
EDA-Fn	extradomain-A fibronectin
EDB-Fn	extradomain-B fibronectin
EGF	epidermal growth factor
EHS	Engelbreth-Holm-Swarm
FA	focal adhesion
FESEM	field emission scanning electron microscopy
FITC	fluorescein isothiocyanate
HCE	human corneal epithelial
HPV	human papilloma virus
Kb	Kilo-base pair
kD	kiloDalton
Lm	laminin
Lu	Lutheran
MAb	monoclonal antibody
MMP	matrix metalloprotease
Onc-Fn	oncofetal fibronectin
PBS	phosphate-buffered saline
PC	polyclonal
PMSF	phenylmethylsulfonyl fluoride
RGD	arginine-glycine-aspartic acid
RIPA	radioimmunoprecipitation
RPMI	Roswell Park Memorial Institute
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
Sol	soluble
SV40	Simian virus 40
Tn-C	tenascin-C
$Tn\text{-}C^{L}$	large subunit of tenascin-C
TNS	trypsin neutralizing solution
TRITC	tetramethylrhodamine isothiocyanate

3. ABSTRACT

The repair of corneal wounds requires both epithelial cell adhesion and migration. Basement membrane (BM) and extracellular matrix (ECM) proteins function in these processes via integrin and non-integrin receptors. We have studied the adhesion, spreading and migration of immortalized human corneal epithelial (HCE) cells and their interactions with the laminins, fibronectins and tenascins produced.

Among laminin (Lm) chains, the α3 and α5 chains were detected in the BM of the human cornea, while Lm α1 was not found in these experiments. This result showed that human corneal BM expresses Lms-332 and -511. HCE cells produced Lm α3', α3, β3, γ2' and γ2 chains to the culture medium, whereas neither Lm-111 nor Lm-511 was produced. Both Lm γ2' and γ2 chains were also found in a cell-free ECM preparation of HCE cells. Because HCE cells did not produce Lm-511, although it was present in corneal BM, we suggest that Lm-511 is produced by stromal keratocytes.

The adhesion of HCE cells to Lms-111, -332 and -511 was studied first by determining the integrin and non-integrin receptor composition of HCE cells and then by using quantitative cell adhesion assays with function-blocking monoclonal antibodies (MAbs). Immunofluorescence studies revealed the presence of integrin α2, α3, α6, β1 and β4 subunits. The cells adhered via integrin α3β1 to both purified human Lms-332 and -511 as well as to endogenous Lm-332. Although several available function-blocking MAbs to integrin α-subunits were tested, we could only demonstrate the role of integrin β1 subunit in HCE cell adhesion to mouse Lm-111. Among the non-integrin receptors, Lutheran (Lu) was found by Northern blot analysis as 4.0 and 2.8 kb mRNA transcripts. This receptor was located to the basal aspect of basal corneal epithelial cells in vivo and as punctate reactivity on the cell surfaces of adhering HCE cells. On the other hand, α- and β-dystroglycans were not present in cornea or HCE cells. The adhesion of HCE cells to Lm-511 was mediated by Lu as well as by integrin α3β1. Since the adhesion of HCE cells to Lm-511 did not induce Lu into focal adhesions (FAs), we suggest that Lm-511 serves as an ECM ligand enabling cell motility.

HCE cells produced and deposited extradomain-A fibronectin (EDA-Fn), oncofetal fibronectin (Onc-Fn) and tenascin-C (Tn-C), which are also found during corneal wound healing. Furthermore, the results showed that Tn-C is deposited vectorially since it was only found in ECM. EDA-Fn and Onc-Fn by contrast, were present both in ECM and in culture medium of HCE cells. Integrin α5β1, which was found in FAs and cell surface-associated ECM adhesions in HCE cells, mediated the adhesion of HCE cells to Fn. However, during the adhesion, integrin α5β1 functioned in concert with integrin αvβ6 and both were localized in FAs. Arginine-glycine-aspartic acid (RGD) peptide inhibited the adhesion of HCE cells to fibronectin as well as to vitronectin. Integrin αvβ6 appeared to be the receptor mediating HCE cell adhesion to vitronectin. Although the cells did not adhere to Tn-C, they adhered to the Fn/Tn-C coat and were then more efficiently inhibited by the function-blocking MAbs against integrin α5 and β1 subunits as well as RGD peptide.

During the early adhesion, HCE cells codeposited Lm-332 and the large subunit of tenascin-C (Tn-C^L) as a restricted plaque beneath the cells via the Golgi apparatus and microtubules. Integrin β4 subunit, which is a hemidesmosomal component, did not mediate the early adhesion of HCE cells to Lm-332 or Lm-332/Tn-C. Based on these results, we suggest that the adhesion of HCE cells is initiated by Lm-332 and modulated by Tn-C^L, as it has been reported to prevent the assembly of hemidesmosomes. Thereby, Tn-C^L functions in the motility of HCE cells during wound healing. The different distribution of Lm-332 and Lm-α3'32 in adhering, spreading and migrating HCE cells suggests a distinct role for these isoforms. We conclude that the processed Lm-332 functions in cell adhesion, whereas the unprocessed Lm-3'32 participates in cell spreading and appears to be produced by HCE cells starting to migrate in experimental wounds.

4. INTRODUCTION

The cornea functions as an optical lens that transmits and focuses light to the retina. This role requires that it is transparent and sufficiently curved. Thus, the cornea has a unique structure exhibiting several special characteristics, such as the specific composition and organization of stromal collagen fibrils (Meek and Boote, 2004), avascularity and rigidity. Refractive surgery has been one of the major topics in the field of ophthalmology during recent years. Understanding of the restoration of corneal structure after corneal wounding is important in the development of new treatments for corneal epithelial defects and to obtain best surgical outcomes.

Corneal wound healing follows a similar pattern to wound healing of the skin and other stratified squamous epithelia of body tissues, although variations exist in the extent of these processes. Characteristic features of corneal wound healing are the complex cellular interactions between epithelial cells and stromal keratocytes mediated by cytokines and growth factors and leading to a non-fibrotic healing process (Fini and Srramer, 2005; Wilson et al., 1999). These stromal-epithelial interactions mediate such processes as corneal epithelial cell migration, proliferation and differentiation, all of which are required for proper re-epithelization of a defective cornea (Suzuki et al., 2003). Prior to cell migration, ECM proteins, such as fibronectin and tenascin-C, are secreted to the wound surface (Tanaka et al., 1999; Tervo et al., 1991a). Specific cell membrane structures, including e.g. filopodia, lamellipodia and focal contacts, which connect the ECM proteins via cell surface receptors to intracellular cytoskeletal fibres, participate in cell migration (Small and Resch, 2005; Small et al., 1996). Normally after photorefractive keratectomy, the wound area is covered by an adjacent intact epithelial cell monolayer and is followed by cell proliferation and restoration of normal epithelial thickness in two to three days (Fagerholm, 2000). This early phase in wound healing serves not only to recover vision but is also important as a barrier against infections. The restoration of corneal epithelium, is not however completed for some weeks, until the permanent anchoring structures consisting of hemidesmosomes, desmosomes and tight as well as adherens junctions are established (Suzuki et al., 2003). Stromal regeneration, which involves a variety of cells, continues for months. While some of the stromal keratocytes undergo early-phase apoptosis (Laube et al., 2004; Li et al., 2000; Netto et al., 2005; Wilson, 2000), others proliferate, migrate or transform into myofibroblasts (Kuo, 2004). Stromal remodelling includes production and reabsorption of collagen and production of glycosaminoglycans by fibroblasts and myofibroblasts (Kuo, 2004). Inflamatory cells, including polymorphonuclear leukocytes and monocytes/macrophages, invade the wound area via limbal vessels and tear fluid (Fagerholm, 2000; Mohan et al., 2003a; Wilson et al., 2004). These cells are responsible for cleaning the wound of cell debris and bacteria. The wound contraction is accomplished by myofibroblasts (Jester et al., 1995).

BM demarcates corneal epithelial cells from Bowman`s layer as a thin layer of ECM. Laminins are heterotrimeric glycoprotein components of BMs that participate in several essential processes, such as cell adhesion, proliferation, migration and differentiation (Colognato and Yurchenco, 2000). Laminins function in these cellular activities by

utilizing transmembrane cell surface receptors. Integrins, which mediate bidirectional signalling between the ECM and the intracellular cytoskeleton, are the best-characterized receptors of laminins and other ECM proteins (Hynes, 2004). Fibronectin is expressed widely in the cornea (Tuori et al., 1996) and is able to promote cell adhesion (Ruoslahti, 1988), whereas tenascin-C (Tn-C) is restricted to the limbal area (Maseruka et al., 2000) and has been considered to modulate cell adhesion (Murphy-Ullrich, 2001; Orend and Chiquet-Ehrismann, 2000).

To investigate the dynamics of corneal epithelial cells during adhesion and wound healing, cultured cells are an obvious choice. There are several methods to culture and propagate primary HCE cells isolated from tissues. However, the problem is that usually the cells can be propagated only for a few generations and pieces of corneas available for culture are scarce. Immortalized corneal epithelial cells have been established with an extended culture life. In these studies, immortalization either by Simian virus 40 (SV40) (Araki-Sasaki et al., 1995; Kahn et al., 1993; Offord et al., 1999) or human papilloma virus (HPV)-16 genome (Mohan et al., 2003b) have been used. Among these models SV40 transformed HCE cells have been most widely used. Primary cultures of HCE cells were for this purpose infected by Dr. Araki-Sasaki and colleagues (1995) with a recombinant SV40 adenovirus vector and were cloned three times to obtain a continuously growing cell line that does not shed free viruses. HCE cells succeeded in growing for more than 400 generations and exhibited a typical cobblestone-like appearance, resembling that of primary corneal epithelial cells in culture. Both the original study and studies thereafter (Huhtala et al., 2003) have shown that these cells express cornea-specific keratins together with some simple epithelial cell cytokeratins not found in the cornea in vivo. Moreover, HCE cells were shown to differentiate in a multilayered fashion at the air-liquid interface. Further studies have revealed HCE cells to be a promising corneal substitute for drug delivery research (Toropainen et al., 2003; Reichl et al., 2004).

In this work, the distribution of laminin isoforms in human corneal BM and their synthesis by HCE cells was studied. Furthermore, the production and deposition of fibronectin and Tn-C by HCE cells was investigated. To obtain more information on the adhesion characteristics of HCE cells to these BM and ECM proteins, the integrin and non-integrin receptors of HCE cells were also studied. Corneal wound healing involves epithelial cell adhesion, spreading and migration, all of which were considered in this thesis.

5. REVIEW OF THE LITERATURE

5.1 Extracellular matrix

Tissues are composed of cells and surrounding networks of macromolecules forming the ECM. The amount and the organization of these macromolecules vary, reflecting the function of each tissue. The epithelial tissues rest on a thin sheet of specialized ECM, called the BM. In the connective tissue, the cells are embedded among ECM molecules and ground substance. The major elements of ECM are fibrillary proteins, collagens and elastins, non-fibrillary proteins, such as fibronectins and tenascins, and BM molecules, including laminins, collagen IV and nidogen. The ground substance consists of glycosaminoglycans, proteoglycans and proteoglycan aggregates. ECM molecules interact with cells via cell surface receptors, affecting cell survival, differentation, migration, proliferation and shape (Adams and Watt, 1993; Hagios et al., 1998). Additionally, ECM resists compressive forces, permits diffusion of nutrients, metabolites and hormones and serves as a reservoir for growth factors and cytokines. As ECM is a dynamic structure, there is a regulated continuous remodelling of these macromolecules.

5.2 Basement membrane – a dynamic structure with diverse functions

BMs consist of specialized ECM molecules that as a thin sheet-like structure underlie epithelial and endothelial cells, separating them from connective tissue. In addition, BMs encircle individual fat cells, muscle cells and Schwann cells. Although the existence of BM has been proposed since the nineteenth century, only more recently have histochemical, immunohistochemical, biochemical and molecular biological techniques yielded information about its structure and function. Ultrastructural studies have shown three BM zones: lamina lucida, lamina densa and lamina fibroreticularis (for review, see Merker, 1994; specifically for cornea, see Beuerman and Pedroza, 1996). Our knowledge of the molecular structure of the BM is based first of all on the studies of Nikolas Kefalides and his collaborators (1979), who showed that type IV collagen is a major BM protein. This protein exists in several isoforms and assembles into multilayered networks located throughout the BM (Yurchenco and Ruben, 1988). A major breakthrough in BM research was when laminin was isolated from a mouse Engelbreth-Holm-Swarm (EHS) tumour and shown to be a BM component (Timpl et al., 1979). Timpl and Brown (1996) later established that type IV collagen and laminin networks were connected to each other by nidogen/entactin. Proteoglycans, agrin, perlecan and type XVIII collagen are also ubiquitous structural components of BMs, playing distinct roles in many cellular functions (Iozzo, 2005).

The complex structure of BMs not only provides mechanical stability for cells, but also enables BMs to act in a variety of biological processes. Since the 1980s, this dynamic structure has been shown to be involved in numerous processes in developing embryo and adult tissues (Miner and Yurchenco, 2004; Yurchenco et al., 2004).

5.3 Laminin isoforms – a growing glycoprotein family controlling tissue organization and cellular functions

An antiserum raised against the first laminin reacted with most of the body BMs (Rohde et al., 1979; Timpl et al., 1979). It gradually became clear that laminin is not only one protein; to date 15 different laminin isoforms have been described (Aumailley et al., 2005).

Laminins, which are multidomain trimeric proteins found in all BMs, consist of one α-, one β- and one γ-chain. At present, 5 α-chains, 3 β-chains and 3 γ-chains have been characterized. These chains associate via triple α-helical coiled-coil domains with disulphide bonds and have molecular masses between 140 and 400 kD. Laminins assemble into large crucifix- or T-shaped proteins comprising one long arm and two or three short arms (Aumailley et al., 2005; Miner and Yurchenco, 2004; Patarroyo et al., 2002). Laminin chains to some extent resemble each other and typically consist of domains. The domains are named from the laminin aminoterminus of the Lm-111 molecule as follows: laminin N-terminal globular (LN) domain, laminin epidermal growth factor-like (LE) domain, L4 domain, laminin four (LF) domain, coiled-coil domain, β-knob domain and LG tandem (Aumailley et al., 2005). All these domains are not present in the three laminin chains. Five globular LG modules are arranged in tandem within the C-terminus of all known laminin α chains (Sasaki et al., 1988). These LG modules have been shown to interact with cellular receptors such as integrins, dystroglycans and Lutheran blood group glycoprotein (Lu) (Miner and Yurchenco, 2004).

Because of the increase in the number of members of the laminin family, a nomenclature was presented in 1994 to replace the earlier diverse names. In this system, laminins were numbered with arabic numerals according to the order of discovery. The genes for laminin chains were named LAMA, -B and -C for α-, β- and γ-chains, respectively (Burgeson et al., 1994). A new simple laminin nomenclature has now been introduced to reveal the chain composition of each laminin (Aumailley et al., 2005). This laminin nomenclature and chain composition are presented in Table 1 and are used in this review.

The structural diversity of laminins enables highly specialized functions. Laminins provide a substratum for cell adhesion and migration and play a role in cell proliferation, differentiation, filtration and cell survival (Chen and Strickland, 2003; Colognato and Yurchenco, 2000; DeHahn et al., 2004; Ekblom et al., 1980; Frank and Carter, 2004; Nguyen et al., 2000). The expression of laminins is regulated spatially and temporally in a tissue-specific manner (Fleischmajer et al., 2000; Sanes et al., 1990; Virtanen et al., 1995; 1996). In recent years, mutations in laminin genes have been found to cause several organelle dysfunctions. In addition, the generation of knock-out mice for laminin chains has provided new information about their specific functions and new models for human diseases (Miner and Yurchenco, 2004).

Both laminin α1 chain- and γ1 chain-deficient mouse embryos die during the early postimplantation period after embryonic day 6.5 or 5.5 due to failure of endoderm

differentiation and BM formation, respectively (Alpy et al., 2005; Miner et al., 2004; Smyth et al., 1999). Also homozygous Lm α5 -/- mice suffer from multiple defects in tissue morphogenesis and differentiation and do not survive past embryonic day 15 to 17 (Miner et al., 1998). On the other hand, β2 chain deficiency does not play an important role during early embryonic development, but such mice have defects in renal glomerular filtration and neuromuscular junctions as well as ocular abnormalities, including microcoria, and the mice die postnatally (Miner et al., 2006; Noakes et al., 1995a; b). Particularly muscular defects in Lm β2 chain mutant mice correlate with the severe failure-to-thrive phenotype (Miner et al., 2006). In nephrotic mutant mice, Lm β2 chain was structurally replaced by β1 chain, but the glomeruli did not function properly, reflecting the highly chain-specific functions of laminins (Noakes et al., 1995a). In man, mutations in LAMB2 gene may cause congenital nephrotic syndrome with microcoria (Pierson´s syndrome; see Zenker et al., 2004; 2005; VanDeVoorde et al., 2006), or nephrotic syndrome with or without minor ocular changes (Hasselbacher et al., 2006). Thyboll et al. (2002) discovered that laminin α4-null mice show extensive bleeding and deterioration of microvessel growth, demonstrating a central role for this laminin chain in microvessel organization. Some muscular dystrophies have been found to be associated with laminin chain deficiencies such as merosin-deficient (Lm α2-deficient) congenital muscular dystrophy (Helbling-Leclerc et al., 1995; Hillaire et al., 1994; Tome et al., 1994). Indirect immunofluorescence studies have shown that the expression of Lm-332 is defective in the epithelial BMs of patients with junctional epidermolysis bullosa of Herlitz`s type (Meneguzzi et al., 1992). Furthermore, several studies have revealed that mutations in genes encoding laminin α3, β3 and γ2 chains are involved in the pathogenesis of the aforementioned disorder, resulting in blistering (Kivirikko et al., 1995; 1996; Pulkkinen and Uitto, 1999). Lm-332 together with Lm-511 will be discussed in more detail in Sections 5.3.1 and 5.3.2.

Table 1. New laminin nomenclature

Chain composition	Current name	Previous name
α1β1γ1	Laminin-111	Laminin-1, EHS-laminin
α2β1γ1	Laminin-211	Laminin-2, merosin
α1β2γ1	Laminin-121	Laminin-3, s-laminin
α2β2γ1	Laminin-221	Laminin-4, s-merosin
α3β3γ2	Laminin-332	Laminin-5, kalinin/nicein/epiligrin
α3β1γ1	Laminin-311	Laminin-6, k-laminin
α3β2γ1	Laminin-321	Laminin-7, ks-laminin
α4β1γ1	Laminin-411	Laminin-8
α4β2γ1	Laminin-421	Laminin-9
α5β1γ1	Laminin-511	Laminin-10
α5β2γ1	Laminin-521	Laminin-11
α2β1γ3	Laminin-213	Laminin-12
α3β2γ2	Laminin-322	Laminin-13
α4β2γ3	Laminin-423	Laminin-14
α5β2γ3	Laminin-523	Laminin-15

5.3.1 Laminin-332

Lm-332 was described independently by several research groups as BM 600 (Verrando et al., 1987), kalinin (Rousselle et al., 1991), epiligrin (Carter et al., 1991), nicein (Marinkovich et al., 1993) and ladsin (Miyazaki et al., 1993). Numerous studies have thereafter demonstrated that this protein participates in different processes, including epithelial cell adhesion, spreading and migration (Hintermann and Quaranta**,** 2004**;** Nguyen et al., 2000). The deposition of precursor Lm-332, which interacts with integrin α3β1, has been shown to promote polarization and migration of human primary keratinocytes in cell culture (Frank and Carter, 2004) as well as in epidermal wounds (Nguyen et al., 2000). On the other hand, Lm α3 chain was also shown to function in the induction and maintenance of cell adhesion and to mediate hemidesmosome formation (Baker et al., 1996). These opposite roles of Lm-332 have been explained by the processing of LG domains of α3 chain, with only the processed α3 chain appearing to induce hemidesmosome formation and cell adhesion via integrin α6β4 (e.g. Hintermann and Quaranta, 2004).

The Lm α3 chain exists in two chain variants, α3A and α3B, due to alternative splicing (Airenne et al., 2000; Aumailley et al., 2003). Therefore, Lm-332 is a T-shaped or cruciform molecule with truncations in all three short arms. The N-terminal region of the short splice variant α3A contains only laminin-type epidermal growth factor-like domains LEc1-3, the β3 chain has six LE domains and one globular LN domain, and the γ2 chain has six LE domains and one globular L4m domain (Aumailley et al., 2003). By contrast, the full-length N-terminal region of the α3B chain consists of additional LN, LEa, LEb, L4a and L4b domains (Aumailley et al., 2003). The C-terminal region of the unprocessed 200 kD α3 chain folds into five LG domains and is proteolytically cleaved into a 165 kD chain after secretion (Marinkovich et al., 1992). The precursor 155 kD γ2 chain is also subjected to processing of its N-terminal domains, resulting in a 105 kD chain (Marinkovich et al., 1992; Rousselle et al., 1991). The 140 kD β3 chain remains unprocessed.

Based on mRNA expression studies, Kallunki et al. (1992) found γ2 transcript in human fetal skin, lung, kidney, thymus and cerebellum. Galliano et al. (1995), by contrast, described a very tissue-specific expression of laminin α3A and α3B mRNAs in only epithelial tissues, such as skin, tooth germ, respiratory tract, alimentary tract, kidney collecting tubules and also focally in the central nervous system. In another study, Lm α3 mRNA was reported to be strongly expressed during wound repair (Ryan et al., 1994). Kawano et al. (1999) thoroughly studied the localization of Lm-332 in human tissues by immunohistochemistry. Their results showed that Lm-332 is located in the BMs of nearly all epithelial tissues. However, only a cytoplasmic fluorescence for Lm-332 was found in the fundic glands of the stomach and hepatocytes, and it was lacking in the BMs of these structures. The BMs as well as the cytoplasm were negative for Lm-332 in the acini of the pancreas, submaxillary glands and bronchial submucosal glands.

5.3.2 Laminin-511

The first laminin was isolated from a mouse Engelbreth-Holm-Swarm (EHS) tumour (Timpl et al., 1979), and it was called EHS-laminin or laminin-1, and is now known as Lm-111. Several studies with MAb 4C7, which was originally raised against human placental laminin and considered to recognize in human tissues α1 chain of Lm-111 (Engvall et al., 1986), suggested a widespread BM distribution for it in human tissues (Engvall et al., 1990; Sanes et al., 1990; Virtanen et al., 1995). However, further studies with many human tissues, such as kidney, lung and muscle, revealed a restricted mRNA expression for the laminin α1 chain (Vuolteenaho et al., 1994). Additionally, when Miner et al. (1995) cloned and described mouse Lm α5 chain and proposed that it may be a major laminin chain of most adult mouse BMs, suspicions considering the specificity of MAb 4C7 were aroused. The suggestion that MAb 4C7 might cross-react with Lm α5 chain was substantiated in a later study (Miner et al., 1997) in which the authors compared the distribution of all known laminin α-chains (1-5) in mouse tissues. They concluded that in adult mouse tissues Lm α5 chain is the most widely distributed α chain, while Lm α1 chain was found in a much more restricted distribution. They also resolved the ambiguity considering MAb 4C7 and concluded that it recognizes Lm α5 chain in addition to or instead of α1 chain. Finally, during the same year, Tiger et al. (1997) conclusively showed that MAb 4C7 reacts solely with human Lm α5 chain. These findings bear substantial importance; numerous studies conducted with MAb 4C7 had to be re-interpreted and a very wide distribution was suggested for Lm α5 chain in nearly all human adult BMs, with a few exceptions, such as skeletal muscle cells. All epithelial BMs appear to contain Lm α5 chain.

Lm α5 chain is required during embryogenesis and studies with knock-out mice have revealed severe defects, such as exencephaly, syndactyly and placentopathy, leading to lethality late in embryogenesis (Miner et al., 1998). Further studies with α5 -/- mutant mouse embryos and using inducible expression of α5 chain in laminin α5-null mice have shown that it is crucial for hair morphogenesis (Li et al., 2003), distal lung epithelial maturation, VEGF production and lung alveolization (Nguyen et al., 2005), intestinal smooth muscle development (Bolcato-Bellemin et al., 2003) and kidney mesangial cell organization (Kikkawa et al., 2003). Furthermore, Lm-511 has a significant role in cell adhesion, proliferation and migration (Kikkawa et al., 1998; Pouliot et al., 2002; Tani et al., 1999). Lm α5 chain as a component of Lm-521, on the other hand, shows a restricted distribution to synaptic BM and BMs of kidney glomeruli and arterial smooth muscle (Miner and Patton, 1999). Diabetic corneas show a reduced amount of Lm α5 chain in BMs and overexpression of matrix metalloproteinase (MMP)-10, which has been found to degrade several laminin chains (Ljubimov et al., 1998; Saghizadeh et al., 2001).

5.4 Fibronectin

Fibronectin is an ECM glycoprotein that exists in body fluids as a soluble plasma fibronectin and is produced by hepatocytes (Tamkun and Hynes, 1983). It is also found throughout the ECM of connective tissues as an insoluble protein (Stenman and Vaheri, 1978). Fibronectin is a dimer composed of two 250 kD subunits, which are covalently linked near the C-termini by disulphide bonds. Three types of repeating units, each composed of a series of independently folding modular domains, form the fibronectin monomers: type I, type II and type III repeats (Pankov and Yamada, 2002). Fibronectin is encoded by a single gene and exists in multiple forms resulting from the alternative splicing of the primary transcript (ffrench-Constant, 1995; Tamkun et al., 1984). The alternative splicing occurs at three sites: EIIIA (EDA), EIIIB (EDB) and the V (variable in length) region (Kornblihtt et al., 1996; Schwarzbauer, 1991).

In addition to EDA-Fn and EDB-Fn, a third isoform called Onc-Fn exists. This isoform, which is differentially glycosylated (Matsuura et al., 1988; Matsuura and Hakomori, 1985), shows a restricted expression solely in fetal and tumour tissues (Matsuura and Hakamori, 1988). All of the alternatively spliced fibronectin isoforms have a temporally and spatially regulated expression in chicken embryos (ffrench-Constant and Hynes, 1989). In embryonic human tissues, the EDA-Fn isoform is abundant in many BMs, while in adult tissues it is mostly confined to endothelia of larger blood vessels and to smooth muscle cells (Vartio et al., 1987). EDB-Fn has even more restrictive distribution, e.g. in the human kidney (Laitinen et al., 1991), being found in only some BMs of fetal tissues and more generally in developing vessels, and has been suggested to serve as a marker for angiogenesis (Castellani et al., 1994).

The multifunctional fibronectin has been suggested to play a role in cell adhesion and migration during development and wound healing, as well as in many cellular processes such as proliferation, survival, differentiation and blood clotting (ffrench-Constant et al., 1989; Hynes, 1986; Schwarzbauer, 1991). Gene knock-out studies have revealed that the expression of fibronectin is essential for embryogenesis, as gene inactivation causes early embryonic lethality (George et al., 1993). The embryos showed a wide variety of abnormalities. However, much less is known about the specific functions of fibronectin isoforms. Gene knock-out studies have suggested that EDA-Fn is necessary in, for instance, proper epidermal wound healing and normal life span (Muro et al., 2003) as well as in proper motor coordination (Chauhan et al., 2005). In addition, the differentiation of fibroblasts to myofibroblasts, which are required in wound contraction, is regulated by EDA-Fn (Desmouliere et al., 2005). Mice lacking EDB-Fn developed normally, but showed reduced cell growth and impaired fibronectin matrix assembly (Fukuda et al., 2002).

5.5 Tenascin-C

Tenascins (Tns) are a highly conserved family of oligomeric glycoproteins (Jones and Jones, 2000; Hsia and Schwarzbauer, 2005). Tn-C, the first described protein among the five members of the tenascin family (-C, -R, -X, –W and -Y), was discovered in several laboratories in the early 1980s. Tn-C belongs to matricellular proteins, which have a modulatory role in cell adhesion (Orend and Chiquet-Ehrismann, 2000**;** Hsia and Schwarzbauer, 2005; Murphy-Ullrich, 2001). Although Tn-C is highly expressed during development of many tissues, it is only sparsely expressed in adult tissues. However, it becomes re-expressed upon wound healing, inflammation and tumorigenesis (Jones and Jones, 2000).

Tn-C was first considered to be synthesized by mesenchymal cells (Erickson and Bourdon 1989), but is now known to also be produced by epithelial cells (Linnala et al., 1993; Latijnhouwers et al., 1997; Lightner et al., 1994). It was originally described as a six-armed huge macromolecule called a hexabrachion (Erickson and Iglesias, 1984; Jones et al., 1989), assembling in the cells rapidly after translation (Redick and Schwarzbauer, 1995).

The subunits of mammalian Tn-C consist of a amino-terminal oligomerization region, heptad repeats, EGF-like repeats, fibronectin type III repeats and a carboxyl-terminal fibrinogen-like globular domain (Hsia and Schwarzbauer, 2005). Alternative splicing, resulting in the inclusion or exclusion of fibronectin type III repeats, ensures the formation of isoforms (Jones and Jones, 2000; Chiquet-Ehrismann and Chiquet, 2003).

Attempts to elucidate the fundamental functions of Tn-C have been made with knock-out mice studies. A surprising first finding was that Tn-C -/- mice appeared to develop normally (Saga et al., 1992). Further studies with these mice indicated that epidermal wounds and severed nerves also healed in these mice normally (Forsberg et al., 1996). However, abnormal re-innervation of skeletal muscle (Cifuentes-Diaz et al., 2002), normal myelinization but abnormal behaviour (Kiernan et al., 1999) and suppression of hematopoietic activity (Ohta et al., 1998) have been found in these mice. Most of the findings have indicated rather subtle morphological and/or physiological changes that may, however, play an essential role in the survival of an animal (Hsia and Schwartzbauer, 2005).

5.6 Integrins and non-integrin basement membrane and extracellular matrix receptors

During the 1980s many attempts were made to define integral cell surface glycoproteins of nucleated cells that could be considered receptors for ECM proteins, especially for fibronectin. Ruoslahti and his collaborators (Pytela et al., 1985a) first described a 140 kD cell surface glycoprotein that showed properties of a fibronectin receptor, later called integrin α5β1. Integrins today form a large family of related proteins. Currently, 18 α- and 8 β-subunits have been characterized in mammals, forming at least 24 integrin dimers.

These covalently associated subunits bind various ligands such as cell surface proteins, collagens, fibronectins, laminins, Tn-C and vitronectin (Danen, 2005; Hynes, 2004).

Heterodimeric integrins recognize partially cell-type specifically distinct ECM and BM components. The same integrin receptor in distinct cell lines may recognize different ECM proteins (Languino et al., 1989). Ligand binding leads to conformational changes in integrins and activates cytoplasmic signalling pathways (Mould and Humphries, 2004). This signal transduction induces numerous cellular processes, including microfilament dynamics and organization, adhesion complex remodelling, migration, gene expression and cell cycle regulation (Danen, 2005; Hynes, 2002; Martin et al., 2002).

Fibronectin contains specific integrin binding sequences (Plow et al., 2000), one of them being the arginine-glycine-aspartic acid (RGD) tripeptide sequence. This sequence is recognized by integrin α5β1 (Pierschbacher and Ruoslahti, 1984, Pytela et al., 1985a) and also by integrin αvβ3 (Pytela et al., 1985b). In addition, at least integrins α4β1 and αvβ1 bind to fibronectin (Plow et al., 2000). The suggested integrin receptor binding sites for Tn-C are located in fibronectin type III repeats, fibrinogen-like globular domain or EGF-like repeats (Hsia and Schwarzbauer, 2005; Swindle et al., 2001). The RGD sequence, present in the third human and chicken fibronectin type III repeat of Tn-C, has been suggested to mediate adhesion to integrins αvβ3 and αvβ6 (Prieto et al., 1993). Moreover, integrins α2β1 (Sriramarao et al., 1993), α8β3 (Schnapp et al., 1995) and α9β1 (Yokosaki et al., 1994) have been reported to serve as receptors for Tn-C. Furthermore, integrin α3β1 has been considered to be a promiscuous integrin receptor, recognizing different ECM and BM molecules such as laminins, collagens and fibronectins (Plow et al., 2000; Wayner et al., 1988).

Some leucocyte integrins are involved in cell-cell interactions, but most of the integrins function in cell-ECM interactions, mediating bidirectional signals between cells and ECM. At least integrins α2β1 (Pouliot et al., 2000), α3β1 (Kikkawa et al., 1998; Tani et al., 1999), α6β1 (Kikkawa et al., 2000; Tani et al., 1999), α6β4 (Kikkawa et al., 2000; 2004) and αvβ3 (Genersch et al., 2003; Sasaki and Timpl, 2001) have been demonstrated to be receptors for Lm-511, binding to its α-chain at the C-terminal globular domain. Ido et al. (2004) showed that the binding site for integrins α3β1 and α6β1 is the LG3 module of Lm-511. Integrins α6β1, α6β4 (Carter et al., 1991; Nishiuchi et al., 2003) and α3β1 (Ebihara et al., 2000) also serve as receptors for Lm-332.

The non-integrin laminin receptors comprise Lutheran blood group antigen (Lu) (El Nemer et al., 1998; Udani et al., 1998; Zen et al., 1999) and the dystroglycan-glycoprotein complex (Henry and Campbell, 1999). Lu is a transmembrane glycoprotein belonging to the immunoglobulin superfamily. It was found to be overexpressed on the surface of sickle red blood cells (Udani et al., 1998), which tend to adhere to endothelial BMs by binding to the Lm α5 chain (Lee et al., 1998). Kikkawa et al. (2003) have shown that the binding site for Lu is located in the Ln α5 LG3 module and may also require Ln α5 LG1-2 modules. Studies based on Northern blotting and immunostainings revealed that Lu is widely expressed in human tissues, such as fetal liver, placenta and arterial walls (Parsons et al.,

1995), and in mouse tissues (Moulson et al., 2001). Dystroglycan was first discovered as a component of the dystrophin-glycoprotein complex in skeletal muscle and found to be associated with muscular dystrophies such as Duchenne muscular dystrophy (Ervasti et al., 1990; Henry and Campbell, 1999; Ibraghimov-Beskrovnaya et al., 1992). It is a glycoprotein composed of two non-covalently attached components: extracellular α-dystroglycan and transmembrane β-dystroglycan. Dystroglycan provides a linkage between ECM and the cytoskeleton by binding to Lm α1, α2 (Ervasti and Campbell, 1993; Talts et al., 1999) and α5 chains (Ido et al., 2004; Yu and Talts, 2003) as well as to a variety of intracellular molecules (Henry and Campbell, 1999).

Table 2. Integrins and their ligands (excluding leucocyte integrins)

Integrin	**Ligand**
α1β1	Lm-111, collagen
α2β1	Lm-111, Tn-C, collagen
α3β1	Lm-111, Lm-211, Lm-332, Lm-511
α4β1	fibronectin
α5β1	fibronectin
α6β1	Lm-111, Lm-121, Lm-221, Lm-332, Lm-511
α6β4	Lm-111, Lm-211, Lm-221, Lm-332, Lm-511
α7β1	Lm-111
α8β1	fibronectin, Tn-C
α8β3	Tn-C
α9β1	Lm-111, fibronectin, Tn-C
α10β1	collagen
α11β1	collagen
αvβ1	fibronectin
αvβ3	Lm-111, Lm-511, Fn, Tn-C, vitronectin
αvβ5	vitronectin
αvβ6	fibronectin, Tn-C
αvβ8	fibronectin, collagen

5.7 Cell-matrix adhesions

Adhesion of cells to external surfaces is a complex process, involving not only cell surface receptors and extracellular ligands, but also intracellular structures and signalling pathways (Blystone, 2004). Intracellular signalling activity affects gene expression, cell growth, differentiation, proliferation, migration and survival (Wu and Dedhar, 2001). Integrin-mediated cell-matrix adhesions are dynamic structures, having distinct molecular architectures and signalling properties (Berman et al., 2003; Sepulveda et al., 2005). Several factors affect these adhesion structures such as rigidity and molecular composition of the ECM (Ingber, 1997). Studies considering the initial state of cell adhesion reveal that a pericellular hyaluronan coat can mediate early adhesion prior to integrin engagement (Cohen et al., 2003; 2004). Integrin binding to ECM ligands and their clustering trigger

reorganization of actin cytoskeleton and intracellular proteins (Miyamoto et al., 1995; Schoenwaelder and Burridge, 1999), resulting in the formation of cell-matrix adhesions, including focal complexes, focal adhesions (FAs) and fibrillar adhesions. However, binding of integrins to ECM ligands is not sufficient to trigger focal adhesion assembly in many cell types, and additional activation of small GTP-binding protein Rho is required (Bershadsky et al., 2006; Hotchin and Hall, 1995). This activation leads to contraction of the actomyosin system through myosin light chain phosphorylation (Kureishi et al., 1997). Integrins commonly associated with cell-matrix adhesions are α5β1, αvβ3 and αvβ5, which bind to fibrinogen, fibronectin and vitronectin (Geiger et al., 2001). Focal complexes, small punctuating structures located on the edges of the lamellipodium in motile cells, have a unique protein composition (Zaidel-Bar et al., 2003). These structures are transient and may rapidly transform into bigger elongated FAs (Geiger et al., 2001), which are also known as focal contacts. FAs are protein complexes that consist of over 50 known associated proteins, including vinculin, talin, paxillin and many phosphorylated proteins (Zamir and Geiger, 2001). Comparison of fibrillar adhesions with focal complexes and FAs reveals their more central orientation in the cell as well as the association of cytoplasmic tensin, integrin α5β1 and fibronectin fibrils with these adhesion structures (Katz et al., 2000; Zamir et al., 1999; 2000).

5.8 Epithelial cell adhesion and migration – interplay between extracellular matrix and intracellular compartment

Cell locomotion is essential in many physiological as well as pathological processes, including embryonic development, tissue maturation in renewable tissues, wound healing, inflammation and tumour cell invasion and metastasis. Cell migration involves repeated cycles of protrusion of lamellipodia, repeated adhesion to and detachment from ECM ligands (Friedl and Brocker, 2000; Small and Resch, 2005). The protrusions of the polarized cells used in migration are thin filopodia and broad lamellipodia, resulting from the actin polymerization into long parallel bundles or branching networks, respectively (Pollard and Borisy, 2003; Small and Resch, 2005; Welch and Mullins, 2000). The lamellipodia can grow into a particular direction, whereas the filopodia serve as sensors of the local environment (Ridley et al., 2003; Wood and Martin, 2002). Zaidel-Bar et al. (2003) showed that continuous assembly and disassembly of focal complexes, which can transform into focal adhesions, occurred in the advancing lamellipodium of endothelial cells during migration. The contractile actomyosin network has been suggested to induce forces that are involved in pulling of the cell body and trailing the cell edge forward (Lauffenburger and Horwitz, 1996). This process requires the disassembly of the adhesions of the rear edge (Ridley et al., 2003; Small and Resch, 2005). Integrins are major migration-mediating receptors, transmitting mechanical interactions as well as bidirectional signalling between cells and ECM or adjacent cells (Huttenlocher et al., 1996).

5.9 Cornea

5.9.1 Anatomy and histology of the cornea

The cornea, which forms the anterior part of the eye, accounts for most of the eye`s refractive power. To maintain proper vision, the cornea must be avascular and transparent. It consists of five distinct layers, which can be distinguished by light microscope (Figure 1). The outermost layer is the corneal epithelium, in the central cornea comprising five to seven layers of non-keratinized squamous epithelial cells. The number of cell layers increases towards the peripheral cornea, which is continuous with the conjunctival epithelium. The stem cells have been suggested to be located in the junction between these compartments, known as the corneoscleral limbus (Lavker et al., 2004). The limbal stem cell population is the ultimate source for corneal regeneration. Stem cell deficiency leads to abnormalities of the corneal structure, such as vascularization of the cornea, which eventually results in visual impairment or blindness (Dua et al., 2003). The low columnar basal epithelial cells are capable of limited divisions before terminal differentiation and migration towards the ocular surface and finally desquamation. The turnover time is approximately two weeks, reflecting a remarkable ability of the corneal epithelium to regenerate (Cenedella and Fleschner, 1990). To maintain the delicate corneal architecture, the corneal epithelial cells, like other stratified epithelial cells, must display distinct adhesive structures in the lateral and basal cell membranes (Ban et al., 2003; Jamora and Fuchs, 2002; Scott et al., 1997; Van Aken et al., 2000). Tight junctions, adherens junctions and desmosomes between corneal epithelial cells function as barriers and provide mechanical strength between adjacent cells (Ban et al., 2003; Petroll et al., 1999). Hemidesmosomes, on the other hand, reside on the basal aspect of basal corneal epithelial cells, attaching them to the underlying BM (Beuerman and Pedroza, 1996; Gipson, 1992) (Figure 2).

Bowman`s layer underlies the corneal BM and is the superficial part of the stroma. This acellular compartment is composed mainly of fibrils consisting of collagen types I, V, VI and XII (Birk, 2001; Ihanamäki et al., 2004). It has been suggested to represent stromal-epithelial interactions and to lack a critical role in corneal physiology (Wilson and Hong, 2000). The rest of the stroma consists of collagen fibrils, which form lamellae. These lamellae are oriented in precise angles with respect to adjacent lamellae, contributing to the transparency and strength of the cornea. Among collagens, at least types I, III, V, XII and XIII have been found in the corneal stroma (Birk, 2001; Ihanamäki et al., 2004). In addition, the stromal matrix contains several proteoglycans, which are responsible for proper spacing of collagen fibrils as well as for hydration of the stromal matrix (Ihanamäki et al., 2004). Keratocytes are arranged in networks between collagen lamellae, communicating with each other by gap junctions. These fibroblast-like cells produce a stromal matrix during the fetal period and in injuries and also maintain the normal corneal stromal matrix.

The BM of corneal endothelial cells is Descemet`s membrane, which is composed of collagen types IV, VI, VIII, laminins and fibronectins (Ihanamäki et al., 2004). This BM

material is produced by a monolayer of non-regenerating endothelial cells that function in fluid pumping and regulate corneal hydration.

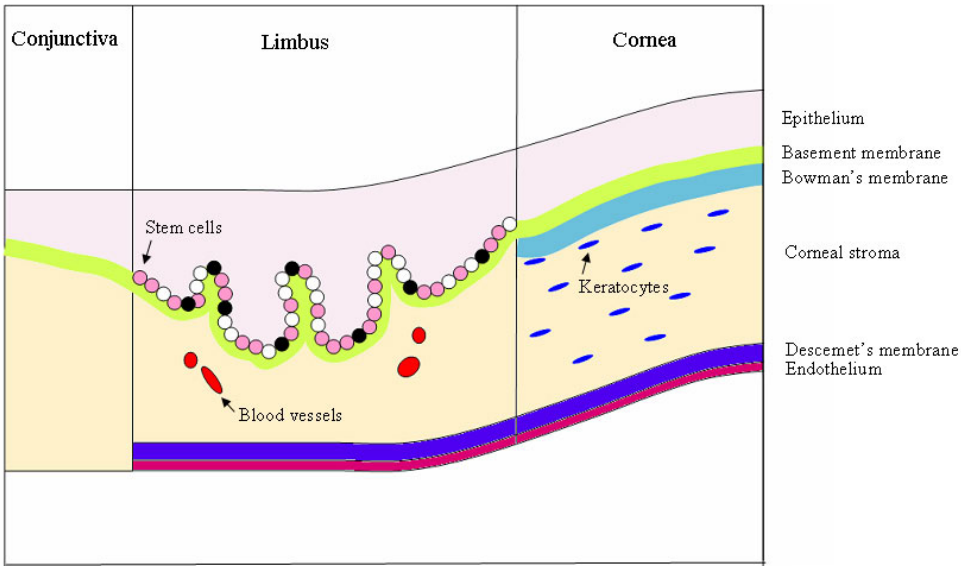

Figure 1. Corneal structure.
A schematic representation of a section through the cornea shows five distinct layers. In addition, the corneal BM is illustrated in the figure. The corneal stroma does not contain blood or lymphatic vessels.

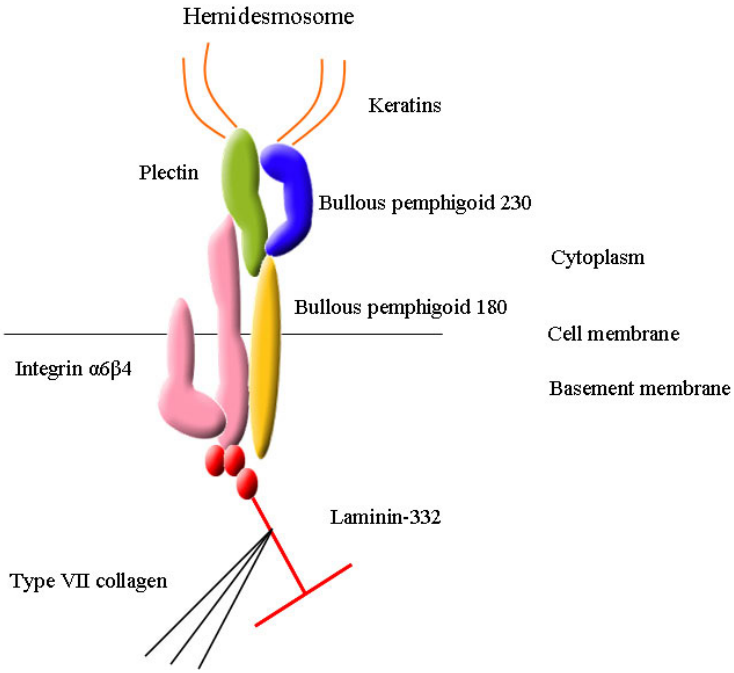

Figure 2. Structure of a hemidesmosome.
This schematic diagram shows the components of the hemidesmosome, which provides an attachment between the basal corneal epithelial cell and the underlying ECM.

5.9.2 Basement membrane and extracellular matrix proteins and their receptors in the cornea

Because corneal BM continues as limbal and conjunctival BM, it would be tempting to assume that the molecular composition of these compartments the same. However, studies on human and animal tissues have shown that the composition of corneal BM differs from that of limbal and conjunctival BM, indicating lateral heterogeneity (Lavker et al., 2004). Of the collagen protein family, which consists of more than 20 distinct collagens, at least collagen XVIII is found in the cornea. Type XVIII collagen was shown to be broadly expressed in mouse ocular tissues, including corneal epithelial BM (Kato et al., 2003). Early studies suggested that BM of the central cornea lacks collagen type IV (Cleutjens et al., 1990; Saika et al., 1995; Tuori et al., 1996). However, collagen type IV exists in six isoforms, and some of these isoforms are also found in corneal BM. Type IV collagen α2 chains are only found in BMs of the conjunctiva and limbus (Fujikawa et al., 1984; Ljubimov et al., 1995; Saika et al., 1999; Tuori et al., 1996). This result was confirmed by Fukuda et al. (1999), who also showed that type IV collagen α5 chains are only found in corneal BM, not in conjunctival BM or the amniotic membrane. However, the laminin composition of corneal and conjunctival BMs was identical. These results suggested that the molecular composition of amniotic membrane and conjunctival BM is the same. However, contrary to the conclusions of Fukuda et al. (1999), Endo et al. (2004) reported that type IV collagen α5 chains are present in both amniotic membrane and corneal BM, implying that amniotic membrane may function as a substrate for corneal epithelial cells.

Adult human corneal BMs were first proposed to be composed of Lm-111 and Lm-332 (Ljubimov et al., 1995; Tuori et al., 1996). The results leading to the assumption that Lm-111 is a component of corneal BM were obtained with MAb 4C7, which is now known to recognize the Lm α5 chain (Tiger et al., 1997). In vitro studies have demonstrated that during corneal wound healing Lm-332 participates in corneal epithelial cell adhesion and migration (Qin and Kurpakus, 1998; Ebihara et al., 2000). While the migration of corneal epithelial cells and keratinocytes has been reported to be mediated by precursor Lm-332 interacting with integrin α3β1 (Ebihara et al., 2000; Nguyen et al., 2000; Frank and Carter, 2004), the processed Lm-332 appears to mediate epithelial cell adhesion via hemidesmosomes containing integrin α6β4 (Goldfinger et al., 1998; Ebihara et al., 2000). Esco et al. (2001) suggested that the loss of processed Lm-332 may play a role in hypoxia-mediated apoptosis of human corneal epithelial cells. Furthermore, a high glucose condition induces the inhibition of Lm-332 synthesis in HCE cells and may correlate to weakened epithelial cell adhesion and manifestation of diabetic keratopathy (Lu et al., 2006).

Fibronectin is also present in corneal, limbal and conjunctival BM zones of human, rabbit and chicken eyes (Päällysaho and Williams, 1991; Tervo et al., 1986; Tuori et al., 1996; 1997b). Of the fibronectin isoforms, EDA-Fn and Onc-Fn have been found in normal human corneal BMs (Tuori et al., 1996; 1997b). Several studies have shown that the expression of fibronectin increases during corneal wound healing (Fujikawa et al., 1981; Nickeleit et al., 1996; Tervo et al., 1991a; Ren et al., 1994; van Setten et al., 1992; Zhao et

al., 2003). PCR studies have substantiated this by demonstrating that the expression of alternatively spliced fibronectin mRNAs are upregulated during rat corneal epithelial wound healing (Cai et al., 1993; Vitale et al., 1994). In addition, fibronectin has been shown to promote epithelial cell migration in the cornea (Nishida et al., 1983).

Tn-C, another multifunctional ECM glycoprotein, has been extensively studied and is found in the normal human and mouse corneal limbus, which is also the site of corneal epithelial stem cells (Tervo et al., 1990; Tuori et al., 1997b; Maseruka et al., 1997; Stepp and Zhu, 1997). Maseruka et al. (2000) showed that Tn-C participates in corneal development since it is expressed widely in the preterm cornea, but is restricted to the limbal area in child and adult corneas. Expression of Tn-C is increased in corneal inflammation, after refractive surgery and during restratification, suggesting a role for this glycoprotein in corneal inflammation, wound healing and ECM reorganization (Maguen et al., 1997; Maseruka et al., 1997; Stepp and Zhu, 1997; Tervo et al., 1991). In addition, its release is significantly increased in tear fluid after photorefractive keratectomy (Tervo et al., 1989; 1991; Stepp and Zhu 1997; Vesaluoma et al., 1995). Despite all of these results, its precise role in the cornea has remained elusive. Iglesia et al. (2000) showed that in Tn-C knock-out mice it is not required for maintainance of the corneal limbus or normal re-epithelization of corneal wounds. Similarly, the healing of cutaneous wounds was normal in Tn-C-deficient mice (Forsberg et al., 1996). Latijnhouwers et al. (1996) concluded that although tenascin is upregulated in skin wounds it is not a substrate for migrating keratinocytes. In healing skin wounds and corneal suture wounds of knock-out mice, however, the absence of Tn-C decreased the expression of fibronectin (Mackie and Tucker, 1999). The correlation between the expression of these ECM glycoproteins was also reported by Matsuda et al. (1999), who showed that in corneal suture wounds of mice Tn-C appears to increase the expression of fibronectin. Overall, the role of Tn-C in normal and wounded corneas has remained unclear and requires further examination.

Vitronectin, a multifunctional glycoprotein, is present in both plasma and ECM and is produced by hepatocytes and many other cells (Schvartz et al., 1999). Vitronectin is also expressed in human corneal BMs and binds to cell receptors by its RGD sequence (Xiao et al., 2005). Since tear fluid also contains this glycoprotein, corneal epithelial cells are exposed to it (Willcox et al., 1997). Vitronectin stimulates corneal epithelial cell migration as well as spreading of keratinocytes, which makes it a suitable subject for wound healing research (Brown et al., 1991; Nakamura et al., 1997).

Integrins α2β1, α3β1, α6β1, α6β4, αvβ1 and αvβ5 are expressed in the human corneal epithelium, mediating the attachment of corneal epithelial cells to the BM and ECM (Rayner et al., 1998; Tuori et al., 1996; Virtanen et al., 1992; for review, see Stepp, 2006). Of these integrins, α6β4 is a component of hemidesmosomes (Borradori and Sonnenberg, 1999), known to mediate the adhesion of HCE cells to Lm-332, in addition to integrin α3β1 (Nguyen et al., 2000; Ebihara et al., 2000). Studies with immortalized bovine and human corneal epithelial cells have shown that these cells are also capable of adhering to mouse Lm-111 and human placental laminin (Lms-511/521) via integrins α3β1 and α2β1 (Kurpakus et al., 1999). In defective human corneas, such as in diabetic and keratoconus

corneas, in vitro and in vivo studies have revealed an altered distribution of integrins α3β1 and α6β4, respectively (Kabosova et al., 2003; Tuori et al., 1997a).

5.9.3 Corneal epithelial wound healing

Adhesive interactions between the cells and the underlying ECM as well as cell-cell interactions are important during corneal wound healing. The basal limbal cells are thought to be stem cells, serving as a source of epithelial cells for the damaged corneal epithelium (Lavker et al., 2004; Zieske et al., 1992). Thus, early proliferative activity upon injury is observed in the limbal area and also among migrating corneal epithelial cells (Dua and Forrester, 1990; Ratkay-Traub et al., 2001). However, Zagon et al. (2000) discovered that re-epithelization of the corneal epithelium is particularly dependent on mitosis in the unwounded epithelium adjacent to the wound. To fully recover the corneal structure, epithelial cells migrate and cover the defective area, proliferate and differentiate (Suzuki et al., 2003). These processes are regulated by cytokines and growth factors, which are produced locally (Tervo et al., 1997; Vesaluoma et al., 1997; Wilson et al., 1994).

After photorefractive keratectomy, the complete coverage of the corneal wound by epithelial cells is completed within 48-72 hours (Fagerholm et al., 2000). However, permanent anchoring requires a much longer time. Fountain et al. (1994) showed that after excimer keratectomy the human corneal anchoring fibrils do not recover completely even after 15 months. Certain clinical conditions, such as viral infections or diabetic keratopathy, can markedly alter normal wound healing. Corneal wound healing can be divided into three phases: latent phase, healing phase, including cell migration and proliferation, and permanent cell adhesion (Dua et al., 1994). In the latent phase, the desquamation of surface cells and the change in the columnar appearance of basal cells to cuboidal form occur. At the end of this phase, the leading wound edge consists of a single cell layer (Crosson et al., 1986). The loss of hemidesmosomes at the marginal cells of the wound is also an early event in wound healing (Ratkay-Traub et al., 2001).

One of the key features in the wound healing process is the synthesis of new proteins. During the latent phase, for instance, the amount of fibronectin increases transiently on the denuded corneal surface (Arffa and Eve, 1991; Fujikawa et al., 1981). Studies concerning refractive surgery show that fibronectin is synthesized after anterior keratectomy (Tanaka et al., 1999; Tervo et al., 1991). Fibronectin functions in the adhesion, spreading, migration and induction of FAs via integrins and is downregulated after wound healing (Fujikawa et al., 1981; Fukuda et al., 1990; Hynes,1992; Mooradian et al., 1993; Nakagawa et al., 1990; Ohji et al., 1993; Suda et al., 1981; Suzuki et al., 2003; Wang et al., 1994). Fibronectin is thus suggested to participate in the wound healing of the cornea (Cai et al., 1993; Murakami et al., 1992; Vitale et al., 1994). The fibronectin receptor integrin α5β1 is upregulated during the healing process (Murakami et al., 1992; Nagakawa et al., 1990). Also Tn-C has been detected in the wound area, although its distribution is normally restricted to the limbus (Latvala et al., 1995; Tervo et al., 1991a). Furthermore, studies on healing alkali burns have shown that type IV collagen emerges into the BM of

the healing corneal epithelium and disappears later (Saika et al., 1995). Both corneal epithelial cells (Ohji et al., 1994) and stromal fibroblasts (Hassell et al., 1992) have been demonstrated to synthesize ECM and BM components in cell culture studies.

The adhesion between corneal epithelial cells is mediated by cell junctions including tight junctions, adherens junctions and desmosomes (McLaughlin et al., 1985). Each of these junctions has a distinct morphology, function and distribution in the stratified corneal epithelium. Gap junctions have been found in basal epithelial cells, tight junctions in superficial cells, desmosomes in wing cells located above basal cells and adherens junctions in all cell layers (Beuerman and Pedroza, 1996; Kapprell et al., 1988; Suzuki et al., 2000; Mohan et al., 1995; Wang et al., 1993**).** Gap and tight junctions as well as desmosomes disappeared from migrating corneal epithelial cells (McCartney and Cantu-Crouch, 1992; Matic et al., 1997; Okada et al., 2001; Suzuki et al., 2000). In order to migrate, corneal epithelial cells must undergo repeated adhesion and de-adhesion cycles. MMPs, which belong to a family of ECM-degrading enzymes, are involved in the degradation of both BM and ECM macromolecules (Birkedal-Hansen, 1995). The expression of MMP-1 and MMP-10 during re-epithelialization of human corneal wounds suggest a role for these MMPs in corneal epithelial cell migration (Daniels et al., 2003). In addition, changes in tear fluid plasminogen-plasmin activity have been reported to correlate with the corneal wound healing process (van Setten et al., 1989). Besides changes in cell junctions, cell-ECM interactions are crucial for corneal wound healing. The BM is often destroyed during corneal injury and the stroma is exposed to the external environment. The strong adhesion of basal corneal epithelial cells to the BM is ensured by hemidesmosomes, which disappear from the zone close to the wound margin (Crosson et al., 1986; Latvala et al., 1996; Kenyon et al., 1977).

In vivo studies on corneal wound healing show that the cells of the leading edge present adherens junctions associated with bundles of actin filament and heal by the contractile "purse string" mechanism (Danjo and Gipson, 1998). However, Buck (1979) observed lamellipodia and filopodia along the leading wound edges. These leading edges have focal contacts. After wound closure, the basal cells proliferate and normal epithelial thickness is restored. Although new hemidesmosomes are established (Stock et al., 1992), Gipson et al. (1989) showed that in the rabbit corneal keratectomy wounds the reassembly of hemidesmosomes did not reach the normal state during the 12-month follow-up period. The corneal epithelial events during wound healing are presented in table 3.

Table 3. Corneal epithelial wound healing

	Latent phase	Healing phase	Permanent cell adhesion phase
Epithelial events	Desquamation of surface cells Single cell layer in the wound edge	Epithelial cell adhesion, spreading and migration Basal cell proliferation	Epithelial cell differentiation Hemidesmosomes assemble

6. AIMS OF THE STUDY

An avascular and clear cornea plays a major role in the refraction of the eye. Several eye diseases, injuries and refractive surgery can produce corneal wounds. Understanding and controlling the wound healing process require knowledge of the complex interactions of corneal epithelial cells with ECM components. The purpose of this study was to gain new information about human corneal epithelial cell interactions with BM and ECM proteins. To achieve this goal, we have investigated the role of laminins, fibronectins, tenascin-C and their integrin and non-integrin receptors in the adhesion, spreading and migration of immortalized human corneal epithelial cells. Specific aims of the study were as follows:

1. To elucidate the laminins present in human corneal BM and to determine those synthesized by corneal epithelial cells.
2. To determine the expression of laminin-, fibronectin-, Tn-C- and vitronectin-binding integrins on corneal epithelial cells and to identify which integrins mediate the adhesion of these cells to mouse Lm-111, human Lms-332 and -511, fibronectin, Tn-C and vitronectin.
3. To study the production and distribution of fibronectin isoforms and Tn-C in corneal epithelial cells.
4. To assess the expression and distribution of non-integrin receptors in corneal epithelial cells and in corneal tissue.
5. To elucidate the role of non-integrin receptors for Lm-511 in adhesion of corneal epithelial cells.
6. To elucidate the role of BM and ECM proteins in early adhesion and migration of corneal epithelial cells.

7. MATERIALS AND METHODS

7.1 Cell culture (I, II, III, IV)

Simian virus-40 immortalized HCE cells were provided by Dr. K. Araki-Sasaki (Dept. of Ophthalmology, Osaka University School of Medicine, Osaka, Japan). HCE cells were cultured in D-MEM/F12 medium (Invitrogen Corp., Carlsbad, CA, U.S.A.) and supplemented with 15% fetal bovine serum, 5 µg/ml insulin (Invitrogen Corp.), 0.1 µg/ml cholera toxin (Sigma, St. Louis, MO, USA), 10 ng/ml human epidermal growth factor (Invitrogen Corp.), 40 µg/ml gentamycin and 1 µg/ml glutamine (Invitrogen Corp.) (Araki et al., 1993). The cultures were maintained in 95% air and 5% CO_2 at 37°C, and the cells were subcultured twice a week. In some experiments, the cells were exposed to 1-10 µM monensin (Lilly Research Laboratories, Indianapolis, IN, USA) for 1-2 h to overnight to stop secretion and induce the intracellular accumulation of secretory products (Tartakoff, 1983).

Jar human choriocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and human embryonic skin fibroblasts from a local source. Both of these cell lines were grown in RPMI-1640 medium (Sigma), supplemented with 10% fetal bovine serum and antibiotics.

7.2 Human corneas (I, III)

Human corneal tissues were obtained from cadaver donors (Helsinki University Central Hospital, Helsinki, Finland), from the Department of Forensic Medicine (University of Helsinki, Helsinki, Finland; specimens kindly provided by Dr. Antti Tuori) or from penetrative keratoplasty performed at the University Hospital of Oulu (Oulu, Finland; specimens kindly provided by Dr. Marko Määttä). The central parts of the cadaver corneas were used in transplantation and only the peripheral parts were included in our studies. The specimens were frozen in liquid nitrogen and stored at -80ºC. Frozen sections were cut to 5-6 µm, fixed in acetone, precooled to -20ºC for 10 min and subjected to immunohistochemistry. The study protocols were approved by the local ethics committees, and patients signed an informed consent.

7.3 Indirect immunofluorescence technique (I, II, III, IV)

For indirect immunofluorescence experiments, HCE cells were first grown on glass coverslips and then fixed in either methanol at -20°C or 4% paraformaldehyde at room temperature for 10 min. After washing three times with phosphate-buffered saline containing 0.1% sodium azide (PBS azide), the cells were soaked in 0.125% Triton X-100 in PBS azide and incubated with primary antibody for 30 or 60 min. All of the antibodies, including their specificity and sources, are listed in Table 4. The MAbs and PC antibodies were applied at a concentration of 2-4 µg/ml. The cells were washed again three times with PBS azide and soaked in 0.125% Triton X-100 with PBS azide. Finally, secondary antibody was added for another 30 min. The following secondary antibodies were used: fluorescein isothiocyanate (FITC)- coupled goat antiserum against mouse or rat IgG, FITC-coupled rabbit antiserum against mouse or rat IgG, tetramethyl rhodamine isothiocyanate (TRITC)-coupled sheep antiserum against rabbit IgG and TRITC-coupled goat antiserum against rabbit or mouse IgG (all from Jackson Immunoresearch; West Grove, PA, USA). Additionally, in some experiments, Alexa Fluor-labelled goat antiserum against mouse (488) or rabbit IgG (594) was used (Molecular Probes, Eugene, OR, USA). All incubations were carried out at room temperature. Finally, the cells were embedded in Veronal-glycerol buffer (1:1; pH 8.4) or in 90% glycerol/10% Veronal buffer if subjected to confocal laser scanning microscopy. The cells were examined with a Leica Aristoplan microscope or an Olympus Provis fluorescence microscope, and the images were acquired using Analysis software (Soft Image Systems, Muenster, Germany) on a computer connected to a SensiCam 12 bit cooled imaging digital camera mounted on the microscope. Confocal laser scanning microscopy (CLSM) was carried out using a Leica TCS SP2 system with an argon excitation wavelength 488 nm and an HCX PL APO CS 63x1.40 NA oil immersion objective. Image stacks were collected through the specimen using a standardized 120 nm z-sampling density. Selected image stacks were further subjected to deconvolution and restoration using theoretical point spread function and iterative maximum likelihood estimation algorithm (Scientific Volume Imaging BV, Hilversum, the Netherlands).

Table 4. Antibodies and molecular probes used

Antibody	Antigen specificity	PC/MC	Dilution	Reference	Source
Anti-hLN-α 1G4/G5	Laminin α1-chain	PC		Tiger et al., 1997	D. Gullberg
163DE4	Laminin α1-chain	MC		Virtanen et al., 2000	
161EB7	Laminin α1-chain	MC		Virtanen et al., 2000	
BM2	Laminin α3-chain	MC, FB	1:200	Rousselle et al., 1991	P. Rousselle
12C4	Unprocessed laminin α3-chain	MC		Goldfinger et al., 1999	J. Jones
4C7	Laminin α5-chain	MC		Engvall et al., 1986; 1990 Tiger et al., 1997	E. Engvall
6F12	Laminin β3-chain	MC		Marinkovich et al., 1992	R. E. Burgeson and P. Rousselle
Pc Ln-5	Laminin-332	PC		I, this thesis	
TS 2/7	Integrin α1	MC		Hemler et al., 1984	M. E. Hemler
CLB-10G11	Integrin α2	MC		Giltay et al., 1989	Netherlands Red Cross Blood Transfusion Service
PIE6	Integrin α2	MC, FB	1:500	Wayner et al., 1988	Chemicon, Temecula, CA
J143	Integrin α3	MC		Fradet et al., 1984	L. J. Old
PIB5	Integrin α3	MC, FB	1:500	Wayner et al., 1984	Chemicon Temecula, CA
B1E5	Integrin α5	MC, FB	1:5	Werb et al., 1989	Z. Werb
GoH3	Integrin α6	MC, FB	1:400	Sonnenberg et al., 1987	Netherlands Red Cross Blood Transfusion Service
Y9A2	Integrin α9	MC		Wang et al., 1996	
LM 142.69	Integrin αv	MC		Cheresh and Spiro, 1987	D. A. Cheresh
PIF6	Integrin αvβ5	MC, FB	1:500	Wayner et al., 1988	Chemicon Temecula, CA
E7P6	Integrin αvβ6	MC, FB	1:500	Weinacker et al., 1994	Chemicon Temecula, CA

102DF5	Integrin β1	MC		Ylänne and Virtanen, 1989	
13	Integrin β1	MC, FB	1:500	Yamada et al., 1990	
90BB10	Integrin β3	MC		Ylänne et al., 1988	
3E1	Integrin β4	MC		Hessle et al., 1984	Chemicon Temecula, CA
AA3	Integrin β4			Tamura et al, 1990	V. Quaranta
ASC-3	Integrin β4	MC, FB	1:500	Pattaramalai et al., 1996	Chemicon Temecula, CA
1A9	Integrin β5	MC		Pasqualini et al., 1993	D. Cheresh
100EB2	Both isoforms of tenascin-C	MC		Howeedy et al., 1990	
BC2	High molecular weight isoform of tenascin-C	MC		Balza et al., 1993	L. Zardi
Tenascin-X	Tenascin-X	PC		Hasegawa et al., 1997	
52DH1	EDA-fibronectin	MC		Vartio et al., 1987	
BC-1	EDB-fibronectin	MC		Carnemolla et al., 1989	L. Zardi
FDC-6	Onc-fibronectin	MC		Matsuura and Hakomori, 1985	S-i Hakomori
TA205	Talin	MC			Serotec, Oxford, UK
IIH6	α-dystroglycan	MC		Ervasti and Campbell, 1993	BD Transduction Laboratories
NCL-b-D6	β-dystroglycan	MC			Novocastra, Newcastle upon Tyne, UK
BRIC 221	Lutheran	MC			Serotec, Oxford, UK
D5B5	Laminin γ2-chain	MC			Chemicon, Temecula, CA; K. Miyazaki
To-Pro-3					Molecular Probes, Eugene, OR
GM130	Golgi protein GM130	MC			BD Transduction Laboratories

PC, polyclonal; MC, monoclonal; FB, function blocking

The concentration of function-blocking antibodies is 1 mg/ml. The dilutions are also provided.

7.4 Field emission scanning electron microscopy (IV)

The cells were cultured on glass coverslips and fixed in 2.5% (w/v) glutaraldehyde (Sigma) in Na-cacodylate buffer (pH 7.2) for 1 h at 4ºC. After fixation, the samples were washed three times with the buffer. After dehydration in an increased series of ethanol, hexamethyldisilazane treatment was used as a final dehydration step (Bray et al., 1993; Nation 1983).

The specimens were mounted on aluminium or copper stubs with double-sided tape and silver glue. Chromium was used as a coating metal (20 μM), and the sputter coating was performed in an EmiTech turbo sputter coater K575-X. All specimens were observed using a JEOL field emission scanning electron microscope (FESEM) JSM-6335F at 5-15 kV (Tokyo, Japan). Some of the specimens were tilted up to 90°.

7.5 Purification of basement membrane and extracellular matrix proteins (I, II)

Affinity chromatography was used to purify Lm-332 from culture medium of BxPC-3 pancreatic adenocarcinoma cells, as described in this section. The antiserum was obtained from rabbits immunized with Lm-332-rich ECM material, which the rat bladder carcinoma cell line 804G deposits (Hormia et al., 1995; Riddelle et al., 1991).

When Lm-332 was immunopurified from the culture medium of SCC25 cells, MAb 6F12 against Lm β3 chain was coupled to the Sepharose, and fibronectin was removed from the medium with gelatin-Sepharose prior to Lm-332 purification. One litre of each culture medium was first precipitated with 40% (NH4) 2SO4 at 4ºC overnight. After centrifugation, the pellets were dissolved in PBS and dialysed against PBS overnight at 4ºC. The Sepharose beads and dialysed culture medium were pooled, and the beads were washed five times with PBS containing 0.5% Triton X-100 and 1 mM phenylmethyl-sulfonyl fluoride (PMSF). Bound proteins were eluated by using glycine buffer (pH 2.8) or acetate buffer (pH 11.3). Fractions containing Lm-332 were pooled and dialysed against PBS.

Human Lm-511 was purified from the culture medium of Panc-1 pancreatic carcinoma cells, which produce only Lm-511 (Tani et al., 1999), as described above. Sepharose-4B-bound MAb DG10 (Virtanen et al., 1997) against Lm β1 chain was used. Lm-511/521 preparation was purchased from Sigma.

Fibronectin and vitronectin were purified from outdated human plasma (Finnish Red Cross Blood Transfusion Service, Helsinki, Finland), fibronectin by using gelatin-Sepharose affinity chromatography (Amersham Pharmacia Biotech, Uppsala, Sweden), essentially according to Engvall and Ruoslahti (1977) and vitronectin by using heparin-Sepharose (Amersham Pharmacia), according to Yatohgo et al. (1988). Purified Tn-C from U251Mg glioma cells was purchased from Sigma.

7.6 Immunoprecipitation, sodium dodecylsulphate polyacrylamide gel electrophoresis and fluorography (I, II, IV)

For immunoprecipitation, HCE cells were incubated 30 min or 2 h in RPMI 1640 medium without serum and methionine and then labelled with ^{35}S-methionine (50-100 µCi/ml; Amersham Biosciences, Uppsala, Sweden) overnight at 37°C. When protein synthesis was studied during the early adhesion of HCE cells, the cells were trypsinized, trypsin-neutralizing solution (Promocell, Heidelberg, Germany) was added and the cells were incubated in methionine-free medium for 1 h. The ^{35}S-methionine labelling was performed for 2 h at 37°C. The culture medium was then collected and the dead cells were removed by centrifugation. Triton-X-100 was added to a concentration of 0.5%. Prior to adding the antibodies, the culture medium was pre-cleared with normal mouse or normal rabbit serum bound to GammaBind Sepharose beads (Amersham Biosciences) to avoid background protein contamination. The cell-free ECM samples were obtained as follows: cell culture plates were washed three times with PBS, put on ice and treated for 3 x 10 min with 0.5% sodium deoxycholate (DOC) in a buffer containing 150 mM NaCl in 10 mM Tris and 1 mM PMSF, pH 8. Finally, the plates were washed with 150 mM NaCl in 2 mM Tris and 1 mM PMSF, pH 8. Solubilization of cells was performed by adding radioimmunoprecipitation (RIPA) salts and 0.1% sodium dodecyl sulphate (SDS). The supernatants were collected, centrifuged for 15 min at 4°C and boiled for 3 min. DOC and Triton-X-100 were added to a concentration of 1%. The clearing was performed as described above. After centrifugation, the supernatants were removed and immunoprecipitated overnight with antibodies prebound to GammaBind^{R} Sepharose^{R} beads. The beads were then washed four times with PBS containing 0.5% Triton X-100 and 1 mM PMSF or RIPA salts. The controls were prepared by precipitating GammaBind® Sepharose® beads only. Finally, the proteins were solubilized in Laemmli's sample buffer and heated 5 min at 90°C. For SDS-PAGE, 5% or 6.5% slab gels were prepared. The gels were loaded with 70 µl of protein samples. High molecular protein weight standards (Amersham Biosciences) were used in all experiments. Finally the gels were dried and subjected to fluorography.

7.7 Western blotting (I, II)

HCE cells were first cultured in serum-free HCE cell culture medium overnight, and ammonium sulphate-precipitated protein samples were then solubilized and separated by SDS-PAGE as described above. The gels were loaded with 100 µl of protein samples. The separated proteins were then transferred to a nitrocellulose membrane electrophoretically and monitored by Ponceau S staining. Remaining hydrophobic binding sites on the membrane were blocked by using 3% BSA in PBS azide or 5% non-fat dried milk in PBS. The membrane was incubated with the primary antibody (2-4 µg/ml) for 1 h at room temperature and washed with 0.01% Tween in PBS. Finally, the biotin-labelled secondary antibody was added to the blot for 30 min, and after washing with 0.01% Tween in PBS, the avidin biotin complex was added (Vectastain^{R}; Vector Laboratories, Burlingame, CA, USA) for 1 h. The bands were detected by using 3,3'-diaminobenzidine (DAB; Sigma), and the reaction was terminated with milli-Q-purified water.

7.8 Northern blotting (III)

Total RNA of HCE cells was isolated by using Trizol® (Invitrogen), which contains phenol and guanidine isothiocyanate, or according to the method of Chomczynski and Sacchi (1987). Trizol, which disrupts the cells and dissolves cell components, was added to the cells prior to addition of chloroform. After centrifugation, the aqueous phase containing the RNA was collected and precipitated with isopropyl alcohol. Finally, the RNA was washed with 75% ethanol and dissolved in distilled DEPC-treated water.

By using Dynabeads Oligo (dt)25-beads (Dynal, Oslo, Norway), mRNA was purified from isolated total RNA. Then 75 µg of total RNA was adjusted to a volume of 100 µl of distilled DEPC-treated water and heated to 65°C for 2 min. The Dynabeads Oligo (dT)25-beads were washed according to the manufacturer`s instructions, and total RNA was added to the Eppendorf tube containing the dynabeads. After a 5-min rotation at room temperature, the supernatant was removed. The dynabeads were washed twice, and elution of mRNA (ca. 2µg) was performed by adding 10 µl of 10 mM Tris-HCl and heating to 85°C for 2 min.

The mRNA samples were prepared by adding 10 x 3-*N*-morpholino propanesulphonic acid (MOPS) running buffer, formaldehyde and formamide, following by incubation at 65°C for 5 min and quick chilling in an ice bath. The mRNAs were then separated according to their size by 1% agarose-formaldehyde gel electrophoresis (Agarose NA; Amersham Biosciences) at 40 V for 3.5 h. Treatment with formaldehyde disrupts the hydrogen bonds between base pairs and denatures the mRNA samples. Upward capillary transfer was used to transfer the mRNAs from the gel to a Hybond N nylon membrane (Amersham Biosciences), and the mRNAs were crosslinked.

A plasmid containing the full-length human Lu coding region was modified as described by Kikkawa et al. (2002). A 700 bp Lu probe was generated by restriction with SmaI (Promega, Madison, WI, USA) and labelled with a Dig High Prime DNA Labelling and Detection Starter Kit II (®Roche Diagnostics, Mannheim, Germany). The DIG-labelled DNA probe was denaturated by boiling for 5 min and cooling in ice. Prehybridization and hybridization were performed at 55°C in high SDS hybridization solution, and the membrane was incubated with the probe/hybridization mixture overnight. Finally, immunological detection was performed, and the membrane was exposed to Hyperfilm MP (Amersham Biosciences). A 0.28-6.58 kb RNA ladder (Promega) was used to determine the transcript size.

7.9 Morphological cell adhesion experiments (III, IV)

For morphological cell adhesion studies, glass coverslips were coated with Lm-511 (4 µg/ml; purified as described in Section 7.5), fibronectin (4 µg/ml; purified as described in Section 7.5), Tn-C (4 µg/ml; Sigma), vitronectin (4 µg/ml; purified as described in Section 7.5) or the combination of these proteins at room temperature for 4 h. The glass coverslips were then washed twice with PBS and postcoated with 3% bovine serum albumin in PBS

at room temperature for 1 h. In some experiments, the cells were incubated with cycloheximide (10 µg/ml; Sigma) at 37°C for 1 h to inhibit protein synthesis. After the addition of trypsin and soybean trypsin inhibitor (STI; Sigma) or trypsin-neutralizing solution (TNS; Promocell), the cells were suspended in serum-free culture medium with or without cycloheximide (10 µg/ml) and centrifuged for 5 min. The supernatant was removed and the cells were suspended in the same medium with or without cycloheximide. Finally, HCE cells were seeded for 1, 2, 3, 4 or 16 h on coated or plain glass coverslips at 37°C. In some experiments, cytochalasin-B (5 µg/ml, Sigma) or demecolcine (5 µg/ml, Sigma) was used to disrupt microfilaments and microtubules, respectively. After the cells were fixed in methanol at -20°C, they were subjected to indirect immunofluorescence.

For in vitro wounding experiments, HCE cells were trypsinized and trypsin-neutralizing solution was added. The cells were washed with D-MEM/F12 medium and suspended in the same medium. HCE cells were seeded and allowed to grow on glass coverslips at 37°C. Confluent HCE cell layers were wounded by scraping with a rubber policeman and allowed to migrate for 12 h.

7.10 Quantitative cell adhesion assays (I, II, III, IV)

Cell adhesion experiments were performed by detecting intracellular acid phosphatase activity (Prater et al., 1991; see Tani et al., 1999). The 96-well plates were coated with laminins, pFn (purified as described in Section 7.5), vitronectin (purified as described in Section 7.5) or Tn-C (Sigma). The applied concentrations of the coating proteins were 2-4 µg/ml. Some wells were left without coating. After a 1-h incubation at room temperature, the wells were washed twice with PBS and some cells were exposed to recombinant soluble-Lu (Sol-Lu; 10 µg/ml; Kikkawa et al., 2002). For postcoating, 3% bovine serum albumin in PBS was added for 1 h, and finally, the wells were washed twice with PBS. Some cells were incubated with cycloheximide (10 µg ml/ml) at 37°C for 60 or 90 min, and all cells were trypsinized and TNS or soybean trypsin inhibitor (Sigma) was added. The cells were washed once with serum-free D-MEM/F12 medium and suspended in the same medium in the presence or absence of cycloheximide. The function-blocking MAbs are listed in Table 4. The MAbs were used at concentrations of 2 - 4 µg/ml to inhibit cell adhesion. Cell-antibody suspension was added to each well containing 20 000 cells and incubated at 37°C for 60-90 min. In a set of experiments, GRGDSP peptide (Sigma) was used at concentrations of 100 - 500 µg/ml to inhibit adhesion. The plates were washed three times with PBS, and 100 µl of the following substrate/lysis solution was added to each well: phosphatase substrate solution (6 mg ml/ml, Sigma) in 50 mM sodium acetate buffer (pH 5) containing 1% Triton-X100. The plates were incubated at 37°C for 1 hr and the reaction was stopped by adding 50 µl of 1 M NaOH to each well. Finally, the air bubbles were removed and the absorbances were measured by an enzyme-linked immunosorbent assay plate reader with a 405 nm filter. Each adhesion experiment was performed with triplicate wells, and standard deviations were calculated to evaluate variations between absorbances.

8. RESULTS AND DISCUSSION

8.1 Corneal distribution and production of laminins by human corneal epithelial (HCE) cells

8.1.1 Laminin composition of adult human corneal basement membrane (I)

Previously, the BM of the human cornea has been considered to express Lms-111 and -332 (Ljubimov et al., 1995; 1998; Tuori et al., 1996; 1997a). We clarified the laminin composition of adult human corneal BM by subjecting frozen sections of human cornea to MAbs against Lm α1, α3 and α5 chains. Immunoreactivities for only Lm α3 and α5 chains were found in these specimens, suggesting the presence of Lms-332 and -511 in human corneal BMs. Our result was obtained with our novel MAbs and also with the same polyclonal (PC) antiserum against the Lm α1 chain that was used in the aforementioned studies. These results are in accordance with earlier ones showing that the Lm α1 chain is not expressed in BMs of human stratified epithelia (Virtanen et al., 2000). This finding is also consistent with the results of Byström et al. (2006) showing that the Lm α1 chain is expressed in BM of fetal human cornea up to embryonic week 12.

8.1.2 Production of laminins and deposition of laminin-332 by HCE cells (I)

The re-estimation of corneal BM laminin composition led us to further investigate the production of laminins by HCE cells. We used an HCE cell line, which resembles normal HCE cells and is easy to maintain (Araki-Sasaki et al., 1995). We did not succeed in properly maintaining in culture normal HCE cells that were repeatedly purchased from commercial sources (Cascade Biologics, Portland, OR, USA). The proteins in the culture medium of radioactively labelled HCE cells were analysed by using immunoprecipitation, SDS-PAGE and fluorography. These experiments with specific MAbs and with PC antiserums showed that HCE cells synthesize unprocessed M_r 190 kD α3 chain (α3'), processed M_r 165 kD Lm α3 chain (α3) and M_r 140 kD Lm β3 chain as well as unprocessed M_r 155 kD Lm γ2 chain (γ2') and M_r 105 kD processed Lm γ2 chain (γ2). In addition, the culture medium of HCE cells and the cell-free ECM preparation were subjected to Western blotting, and antiserum against Lm γ2 chain was used. Again, the presence of M_r 155 kD Lm γ2' and M_r 105 kD Lm γ2 chains were detected. We then studied the deposition of Lm-332 in HCE cell cultures by using the indirect immunofluorescence technique. Already after 1 h of adhesion, Lm-332 was observed as small plaques beneath the adhering cells. After 4 h, Lm-332 was seen as diffuse plaque-like structures in cell cultures. At this stage, a partial colocalization was found for immunoreactivities of Lm-332 and intergrin α6β4.

HCE cells did not, however, produce Lm α1 or α5 chains. For positive control, Jar human chorioncarcinoma cells were also immunoprecipitated. Indeed, these cells produced M_r 400 kD Lm α1 chain and M_r 380 kD Lm α5 chain. These results agree with earlier studies that have shown that epithelial cells produce Lm-332 (Ebihara et al., 2000; Rousselle et al., 1991; Marinkovich et al., 1992; Pierce et al., 1998). Even though Lm-511 was present

in corneal BM and gingival epithelial cells and pulmonary alveolar epithelial cells have been demonstrated to synthesize Lm α5 chain (Pakkala et al., 2002; Pierce et al., 1998), HCE cells did not apparently secrete this laminin. Since earlier studies have suggested that keratocytes might be the origin of corneal BM components (Gipson et al., 1989; Hassell et al., 1992; Tanaka et al., 1999), we proposed that stromal keratocytes synthesize Lm-511.

8.2 Role of laminins and their receptors in HCE cells

8.2.1 Distribution of laminin-binding integrin receptors in HCE cells (I, II)

To analyse the role of corneal laminins, we first had to clarify the integrin receptor repertoire of HCE cells. Integrins are involved in both cell adhesion and migration by binding to BM and ECM proteins and by mediating mechanical forces and regulating the cytoskeletal organization (Ingber, 1997; Sheetz et al., 1998). Earlier studies have shown that the human corneal epithelium expresses integrins α2β1, α3β1, αvβ1, αvβ5, α6β1 and α6β4, which are known to serve as laminin receptors (Lauweryns et al., 1991; Stepp, 2006; Stepp et al., 1990; 1993; Tervo et al., 1991b; Virtanen et al., 1992; Vorkauf et al., 1995). We also found all of these integrins in HCE cell cultures, showing that in this respect HCE cells resemblel normal corneal epithelium. MAb against the integrin α2 subunit reacted heterogeneously with cell surfaces. An overall weak immunoreactivity was seen, yet some cells showed a brighter reaction. Immunostaining for the integrin α3 subunit revealed a more even cell surface-confined immunoreactivity. Both integrin α6 and β4 subunits showed a leopard skin-like immunoreactivity, as reported in previous studies on human keratinocytes (Marchisio et al., 1991). The integrin β1 subunit gave an even cell surface reaction and also confined to nail-like focal adhesions. In addition to integrins and actin terminations, these adhesive contacts also contain various actin-associated and signalling proteins (David et al., 1999; Geiger et al., 2001; Zamir and Geiger, 2001). Immunoreactivity for integrin β3 subunit, a component of laminin-511 binding integrin αvβ3 (Genersch et al., 2003), has not been found in corneal epithelium (Lauweryns et al., 1992; Rayner et al., 1998; Tervo et al., 1991b), and was not observed in HCE cells either.

8.2.2 Expression and distribution of laminin-binding non-integrin receptors in HCE cells (III)

Among the non-integrin laminin receptors, both Lu (El Nemer et al., 1998; Udani et al., 1998) and the dystroglycan-glycoprotein complex (Yu and Talts, 2003) have been shown to serve as receptors for α5 chain laminins. We first explored the expression of these non-integrin receptors in HCE cells and then studied their role in the adhesion of HCE cells.
To determine whether HCE cells express Lu mRNA transcripts, Northern blotting analysis was used. This experiment revealed the presence of both 4.0 kb and 2.8 kb bands of Lu mRNA. The presence of Lu protein was also investigated in human corneas as well as in HCE cells. The peripheral parts of corneas available for this study showed a bright basal cell-confined immunoreaction to Lu. In cultured adhering and subconfluent HCE cells, a punctate diffuse immunoreaction was found for Lu on cell surfaces. The presence of α-

and β-dystroglycan was examined in vivo and in vitro. No immunoreaction was detected with MAbs for these proteins.

8.2.3 Adhesion of HCE cells to laminins (I, II, III)

The adhesion characteristics of HCE cells to laminins were exposed. This is an extremely interesting subject since cell adhesion and de-adhesion are both essential but not very well- studied processes in corneal wound healing. Recently, amniotic membrane transplantation for defective corneas has been an active area of research (Tseng, 2001). This technique is currently used clinically in corneal epithelial ulcerations, limbal stem cell deficiency and chemical burns (Anderson et al., 2001; Arora et al., 2005; Lee and Tseng, 1997). One focus in the field of amniotic membrane transplantation has been its BM composition and the interaction of BM proteins with corneal epithelial cells (Kurpakus-Wheater, 2001).

Purified human Lms-332 and -511 were included in the adhesion experiments since both are present in human corneal BM. The adhesion characteristics to mouse Lm-111 were also investigated. The function blocking MAbs against integrins were chosen according to the integrin composition of HCE cells and previous studies concerning the binding properties of the aforementioned laminins via integrin receptors. Previously, HCE cells have been shown to adhere more efficiently to human placental laminin (Lm-511/521) than to mouse Lm-111 (Kurpakus et al., 1999). Furthermore, Kurpakus et al. (1999) suggested that HCE cells adhere mainly via integrin α3β1, less via integrin α2β1 and not at all via integrin α6β1 to EHS and placental laminins. In contrast, we found that only the integrin β1 subunit mediated the adhesion of HCE cells to mouse Lm-111, while integrin α2, α3 and α6 subunits did not function in this adhesion. Whether a new integrin α subunit exists that mediates HCE cell adhesion to Lm-111 remains to be elucidated. Several studies have shown that epithelial cells adhere to Lm-332 via integrin α3β1 (Carter et al., 1991; Ebihara et al., 2000; Nguyen et al., 2000; Rousselle and Aumailley, 1994). Our results indicated that integrin α3β1 functions in the adhesion of HCE cells to Lm-332, and this integrin mediated the adhesion of HCE cells to Lm-511. Pouliot et al. (2002) have studied the adhesion of human keratinocytes to Lm-511/521. Their results suggest that human keratinocytes utilize both integrin α3β1 and α6β1 in this adhesion. On the other hand, in our study, integrins α2β1 and α6β4 did not function in the adhesion of HCE cells to any of the tested laminins. The results also showed that MAbs against integrins α3 and β1 as well as MAb BM2 against Lm α3 chain partially inhibited the adhesion of HCE cells to newly deposited Lm-332 beneath the cells.

Lu has been reported to be a potent non-integrin receptor for the Lm α5 chain (Kikkawa and Miner, 2005; Kikkawa et al., 2002: Parsons et al., 2001). The adhesion of HCE cells to Lm-511 was evaluated in the presence of cycloheximide, which prevents endogenous protein synthesis. These studies revealed that Lm-511 appears to enhance the spreading of HCE cells. Furthermore, immunoreactivities for Lu and talin were found in the diffuse punctuate pattern on HCE cells adhered to Lm-511. Immunoreactivity for Lu remained cell surface-confined and diffuse in HCE cells adhered to fibronectin or to fibronectin and

Lm-511. On the basis of these results, we suggest that the adhesion of HCE cells to Lm-511 does not induce Lu into FAs. Our experiments under similar adhesion conditions show that fibronectin promotes FA formation, thereby excluding the possibility that cycloheximide would prevent the formation of these adhesion structures. Previous studies suggest that the intermediate state of adhesion includes ECM ligand-induced integrin clustering and cell spreading but not FAs (Murphy-Ullrich, 2001). This state of adhesion is considered to support cell survival and to be the most favourable for cell motility. We therefore suggest that Lm-511 serves as an ECM ligand, enabling HCE cell motility. However, the fact that talin assembled into FAs in HCE cells adhering to Lm-511 together with Fn shows that Lm-511 does not prevent FA formation. Kikkawa et al. (2003) showed that Sol-Lu inhibited the adhesion of mesangial cells to Lm-511 only together with MAb against integrin α3β1 and had only little inhibitory effect on its own. We found that Sol-Lu, which is a recombinant protein containing the Lu-receptor extracellular domain and which saturates the Lu binding site of the Lm α5 chain (Kikkawa et al., 2003), significantly inhibited the adhesion of HCE cells to Lm-511. This inhibition was not enhanced with MAbs against integrin α3 or β1. Our findings can be explained by the binding sites for Lu and integrin α3β1 overlapping in the Lm α5 LG1-3 modules (Kikkawa et al., 2003; Yu and Talts, 2003). Sol-Lu also inhibited the adhesion of HCE cells to the Lm-511/521 preparation, and the effect was increased when cycloheximide was applied to inhibit endogenous Lm-332 synthesis. Sol-Lu failed to inhibit HCE cell adhesion to Lm-332, thereby suggesting that Lu is a specific receptor for the Lm α5 chain.

8.3 Production, secretion and deposition of tenascin-C and fibronectin isoforms by HCE cells (II)

Since the purpose of this study was to clarify the roles of BM and ECM proteins in the cornea, we investigated the production, secretion and deposition of Tn-C and fibronectin by HCE cells. The adhesive properties of fibronectin, vitronectin and the adhesion modulating role of Tn-C were also considered. The deposition of Tn-C and fibronectins by cultured HCE cells was examined by using an immunofluorescence technique. MAbs against EDA- and Onc-Fn yielded a bright cell surface–associated fibrillar immunoreactivity, while immunoreactivity for EDB-Fn was lacking in HCE cells. MAbs against both subunits of Tn-C and Tn-C^L gave a similar diffuse patch-like immunoreaction beneath the cells. Tuori et al. (1999) showed that Tn-X was also present in the corneal BM of rats and humans, but we did not find this glycoprotein in HCE cells.

While studies have revealed that fibronectin is produced by stromal and epithelial cells in healing corneas (Nickeleit et al., 1996; Phan et al., 1989; Vitale et al., 1994), the origin of Tn-C under these circumstances remains unsolved. However, during wound healing of human skin, epidermal keratinocytes express and are a source of Tn-C (Aukhil et al., 1996; Latijnhouwers et al., 1997). To obtain data on the production of fibronectin and Tn-C isoforms in HCE cells, we used Western blot analysis of the culture medium and cell-free ECM. Radioactive metabolic labelling was first applied, but a strong unspecific precipitate with M_r 190-250 kD was consistently found in the gels. In Western blotting, EDA-Fn and Onc-Fn were both present in the culture medium of HCE cells and cell-free

ECM with M_r 240 kD. However, the reaction with MAb against Onc-Fn was much weaker. MAbs against both subunits of Tn-C and Tn-C^L showed a band of M_r 290 kD in cell-free ECM, but not in the culture medium of HCE cells. Furthermore, a weak band of M_r 190 kD was detected in cell-free ECM with MAb against both subunits of Tn-C. ECM produced by human embryonic fibroblasts was used as a control and showed a M_r 240 band with MAb against Onc-Fn. Tn-C purified from the culture supernatant of U251Mg glioma cells was also used as positive control and showed both subunits of Tn-C. These results indicate that HCE cells produce two fibronectin isoforms and Tn-C^L, suggesting that corneal epithelial cells could also produce these ECM glycoproteins in wound healing.

Because Tn-C was only found in ECM, we suggested that it is vectorially deposited and uses different secretory pathway than fibronectin isoforms. This was partially confirmed by exposing HCE cell cultures to monensin, a monovalent ionophore, which stops secretion and induces accumulation of intracellular secretion granules (Tartakoff, 1983). Double immunostaining for fibronectin and Tn-C showed that these ECM glycoproteins appeared to be located to different cytoplasmic granules.

8.4 Role of extracellular matrix glycoproteins and their integrin receptors in HCE cells

8.4.1 Distribution of fibronectin-, tenascin-C- and vitronectin-binding integrins in HCE cells (I, II)

To obtain data for adhesion experiments, we first investigated the content of integrins in HCE cell cultures by indirect immunofluorescence. Previous studies have suggested that corneal epithelial cells would adhere to fibronectin via the following integrins: α3β1, α6β1, α6β4, α9β1, αvβ1 and αvβ6 (for a recent review, see Stepp, 2006). Of these, integrin α9 and β6 subunits were not found in HCE cell cultures. Integrin α5β1 functions as a fibronectin receptor in epidermal keratinocytes (Clark, 1990). Although integrin α5 subunit has only been observed in rat, not human, corneas (Stepp et al., 1993; Tervo et al., 1991b), we identified this integrin subunit in FAs and cell surface-associated ECM adhesions in HCE cells. Studies concerning corneal wound healing show that integrins αvβ6 (Hutcheon et al., 2005) and α9β1 (Stepp and Zhu, 1997) are upregulated during corneal injury. These integrins are known to serve as corneal epithelial cell receptors for vitronectin and Tn-C, respectively (Stepp, 2006). Additionally, vitronectin has been shown to be a ligand for at least integrins αvβ1, αvβ3 and αvβ5 (Plow et al., 2000). The immunoreactivity for integrin α1 and β3 subunits in HCE cell cultures remained negative. MAbs against integrin αv and β5 subunits gave similar reactions, both showing point and peculiar ring-like structures on the cell centres and FAs in the peripheral cells of cell islands in sparce HCE cell cultures. In dense cultures, however, these MAbs showed only ring-like structures on the substratum-adhered side of the cells.

8.4.2 Morphology of the HCE cells adhering to fibronectin, tenascin-C and vitronectin (II)

The spreading of HCE cells as well as their integrin receptors were studied during HCE cell adhesion to ECM proteins by indirect immunofluorescence. For this purpose, HCE cells were seeded on fibronectin-, Tn-C-, vitronectin- and fibronectin/Tn-C-coated substrata. The cells spread on both fibronectin- and fibronectin/Tn-C-coated substrata within 1 h. Less spreading was observed on vitronectin-coated growth substratum, and no spreading on plain Tn-C coating. The latter finding is in line with results showing that cultured keratinocytes adhere poorly to Tn-C, thereby suggesting that Tn-C does not serve as an adhesive substrate for migrating keratinocytes (Latijnhouwers et al., 1997). HCE cells that were plated on plain glass coverslips spread slowly.

Our adhesion experiments showed that integrins α5, β1 and αvβ6 were localized in the FAs within 1 hour in HCE cells adhering to fibronectin. When HCE cells spread on fibronectin/Tn-C coated substratum, integrins α5, αv and β6 remained localized to the FAs. We therefore suggest that Tn-C does not downregulate fibronectin-induced FA formation in HCE cells as it does in fibroblasts (Midwood and Schwarzbauer, 2002). No reactivity was found with MAbs against integrin β3 or β5 subunits on fibronectin or fibronectin/Tn-C coating. Cell surface-confined spots were observed with MAbs against integrin αv and β5 subunits on HCE cells adhered to vitronectin. Previous studies with different cell lines report a similar distribution of integrin αvβ5 on the vitronectin coat (Wayner et al., 1991). Wayner et al. (1991) concluded that the intracellular portion of the β5 subunit does not associate with the actin cytoskeleton or associates with other cytoplasmic proteins and thereby appears as spots. Immunoreactivity for integrin β1, on the other hand, showed a diffuse reaction in HCE cells on the vitronectin coat. Immunoreactivity for integrin α5, α9, β3 and β6 subunits remained negative in HCE cells on this substratum.

8.4.3 Quantitative cell adhesion experiments of HCE cells adhering to fibronectin, tenascin-C and vitronectin (I, II, IV)

Previous studies concerning mostly epithelial cells have suggested that integrin α3β1 would be a promiscuous receptor for fibronectin, laminin and collagen (Plow et al., 2000; Wayner and Carter, 1987). We obtained similar results when we studied the adhesion of HCE cells to fibronectin, which was inhibited with function-blocking antibody against integrin α3β1. However, when cycloheximide was applied to inhibit endogenous protein synthesis during the adhesion process, only MAbs against integrin α5 and β1 subunits inhibited the adhesion to fibronectin, and no inhibition was seen with MAb against integrin α3. The result led us to propose that integrin α3 actually binds to endogenously deposited Lm-332 under normal cell adhesion conditions. This suggestion was supported by earlier observations with human keratinocytes indicating that integrin α3β1 is a receptor for Lm-332 (Zhang and Kramer, 1996) and not for Fn (Eble et al., 1998). We confirmed this hypothesis by allowing HCE cells to secrete and adhere to endogenous Lm-332. Under these circumstances, MAbs against integrin α3 and β1 subunits as well as

MAb BM2 against the Lm α3 chain partially inhibited the adhesion to an uncoated substratum. Evidence supporting the idea that endogenously produced Lm-332 interferes with the adhesion experiments emerged from HCE cells adhering to purified Tn-C only in the absence of cycloheximide. Under these circumstances, the cells adhere to endogenous Lm-332. Although MAb against integrin αvβ6 did not inhibit adhesion to fibronectin alone, the inhibition was nearly complete when MAb against integrin αvβ6 was applied together with function-blocking MAb to the integrin α5 subunit. HCE cells adhered similarly to fibronectin/Tn-C-coated wells as to fibronectin; however, in the presence of Tn-C MAbs against integrin α5 and β1 functioned more effectively. Because both of these ECM proteins are produced during wound healing, we suggest that fibronectin and Tn-C cooperate during the adhesion and migration of epithelial cells. However, further studies are needed to thoroughly understand this mechanism. In some adhesion experiments, RGD peptide was used at concentrations of 100 μg/ml and 500 μg/ml. Although RGD peptide (500 μg/ml) inhibited HCE cell adhesion to fibronectin, the inhibition was much higher at both concentrations of RGD peptide when the cells adhered to the fibronectin/Tn-C coated substratum. This result indicates that Tn-C modulates the adhesion characteristics of HCE cells to fibronectin. HCE cells adhered also to the vitronectin-coated substratum, and this adhesion was inhibited with RGD peptide as well as with MAbs against integrin β1 and αvβ5. When cycloheximide was applied, the adhesion-blocking effect of these MAbs and RGD peptide increased significantly. The integrin α subunit that mediates HCE cell adhesion to vitronectin with integrin β1 remained unsolved, most likely however being integrin αvβ1 (Marshall et al., 1995).

8.5 Role of laminin-3'32/332 and tenascin-C in the early adhesion, spreading and migration of HCE cells (IV)

We were particularly interested in the events occurring during early adhesion, spreading and migration of HCE cells, which might reflect true phenomena during corneal wound healing. Numerous studies have shown that Lm-332 functions in these processes in different human epithelial cell lines (Baker et al., 1996; Frank and Carter, 2004; Hormia et al., 1995; Nguyen et al., 2000; Rousselle and Aumailley, 1994) as well as in HCE cells (Ebihara et al., 2000; Qin and Kurpakus, 1998). Kaplony et al. (1991) suggested that also Tn-C^{L} facilitates corneal epithelial cell migration in the embryonic cornea. Furthermore, the amount of Tn-C increases in tear fluid and in corneal stroma during wound healing (Tervo et al., 1991a; Vesaluoma et al., 1995). We have already shown that HCE cells produce both Lm-332 and Tn-C. Whether these proteins are also produced and deposited during the early adhesion of HCE cells was one of the major questions.

8.5.1 Adhering and spreading HCE cells – cell morphology and deposition of proteins (IV)

The morphology of adhering and spreading HCE cells was first studied by using FESEM. The 90° tilting angle of the cell culture combined with a very small adhesion area of roundish cells caused some charging problems during the scanning of the beam. The deposited proteins were detected by indirect immunofluorescence, and the data was

combined with FESEM results according to the adhesion time of HCE cells. After 1 h of adhesion, foot-like processes appeared to protrude from the basal aspect of the cells, and already at this stage a spot-like Tn-C^L plaque was found beneath the adhering cells. The small processes converted into a larger circular area within 1-2 h of adhesion, and small microspikes or filopodia concurrently became attached to the growth substratum. After 1.5 h of adhesion, many of the cells showed asymmetrical lamellipodia-like structures, and also the Tn-C^L plaque was asymmetrically situated beneath the rounded cell body. Eventually, the cells extended wide symmetrical lamellipodia, and a large Tn-C^L plaque was seen beneath the cells. Double immunostaining showed that Lm-332 colocalized with Tn-C^L plaques beneath HCE cells as round or oval plaques during the first 1-2 h of adhesion. After 4 h of adhesion, however, the adhesion plaques were wide, extending diffusely beneath the cells. The colocalization of Tn-C^L and Lm-332 was confirmed by double immunoreaction CLSM images. Based on these results we suggest that both Tn-C^L and Lm-332 functions in HCE cell adhesion.

8.5.2 Adhering and migrating HCE cells – role of laminin-332 isoforms (IV)

Lm-332 isoforms were studied by indirect immunofluorescence to evaluate their role in adhesion, spreading and migration of HCE cells. Previously, Lm-3'32 has been shown to promote keratinocyte and corneal epithelial cell motility via integrin α3β1, while Lm-332 functions in the formation of hemidesmosomes and inhibition of cell migration via integrin α6β4 (Ebihara et al., 2000; Goldfinger et al., 1998; Hintermann and Quaranta, 2004; Marinkovich et al., 1992; Nguyen et al., 2000). Furthermore, Qin and Kurpakus (1998) demonstrated that endogenously secreted Lm-3'32 promotes epidermal growth factor- or transforming growth factor α-stimulated corneal epithelial cell motility. Exogenous Lm-332, however, promoted cell adhesion. The deposition of Lm-332 isoforms by adhering HCE cells was distinct since Lm-3'32 was seen as an even plaque extending from the cell centre to the periphery, but Lm-332 was restricted to the oval plaque beneath the cell body after 2 h of adhesion. The role of Lm-332 isoforms was further studied by immunostaining wounded HCE cell cultures. After an overnight migration, the marginal HCE cells along the wound edge and some of the supramarginal cells showed a bright cytoplasmic immunoreactivity for the Lm α3' chain. Similar results were reported previously with migrating epidermal keratinocytes, which expressed Lm-3'32 at the wound healing front (Lampe et al., 1998; Nguyen et al., 2000). The emergence of cytoplasmic Lm-α3' also in supramarginal cells could indicate the cellular tensegrity forces mediating mechanical information (Ingber, 1997). A bright immunoreactivity was also observed for the Lm β3 chain in the moving marginal cells. Lm α3 chain and Tn-C^L, by contrast, were not found in in vitro wounds, but both were present diffusely in the unwounded HCE cell culture substratum. Based on these results, we conclude that Lm-332 isoforms have distinct roles in the adhesion, spreading and migration of HCE cells. In line with earlier studies, we suggest that Lm-3'32 participates in spreading and migration of HCE cells during corneal wound healing. On the other hand, Lm-332 and Tn-C^L were found as restricted plaques beneath the adhering HCE cells, but not in migrating HCE cells. This result confirms that Lm-332 and Tn-C^L initiate the early adhesion of HCE cells.

8.5.3 Production and secretion of chains of laminin-332 and tenascin-C by early adhering HCE cells (IV)

The synthesis of Lm-332, Lm-3'32, Tn-C and Tn-C^L was studied by using 2-h adhered and S^{35}-methionine-labeled HCE cells. In the ECM specimen, a 200 kD polypeptide, corresponding to the Lm α3' chain, was detected. In addition, a 165 kD polypeptide, corresponding to the Lm α3 chain, a 140 kD polypeptide, corresponding to the Lm β3 chain and 155 kD and 105 kD polypeptides, corresponding to Lm γ2' and γ2 chains, were detected in ECM. The culture medium lacked the Lm α3' chain and very weak bands were obtained with antiserum against Lm-332. MAb against both subunits of Tn-C detected only a 290 kD band in the cell-free ECM preparation, corresponding to Tn-C^L. Some non-specific binding was also seen as high M_r bands. We therefore suggest that adhering HCE cells synthesize both Lm-332 and Lm-3'32 as well as Tn-C^L. This result does not support earlier studies showing that keratinocytes and HCE cells secrete only Lm-3'32, which becomes processed after secretion (Ebihara et al., 2000; Nguyen et al., 2000). However, both Lm-332 isoforms can be secreted depending on the cell line (Goldfinger et al., 1998). Additional evidence supporting adhering HCE cells synthesizing and depositing both Lm-332 isoforms lies in their different distribution beneath the early adhering HCE cells.

Furthermore, our results suggest that most Tn-C^L and Lm-3'32 are directly delivered to the ECM. This result prompted us to further investigate the secretion and deposition of Lm-332 and Tn-C^L. First, the position of the Golgi apparatus was determined in 2-h adhered HCE cells with immunofluorescence by using MAb GM130 specific for a Golgi protein, and CLSM imaging. The immunoreactivity for the Golgi apparatus was located to the vicinity of the deposited Lm-332 plaque. Thereafter, the cells were exposed to demecolcine over a 2-h adhesion. As a result of this disrupting treatment for microtubules and the Golgi apparatus, immunoreactivities for Lm-332 and Tn-C^L plaques were not found beneath the adhering HCE cells. Instead, both proteins were observed diffusely around and beneath the HCE cells. A different pattern was seen when HCE cells adhered in the presence of cytochalacin-B, which disrupts the microfilaments. Now both Lm-332- and Tn-C^L-immunoreactive plaques were found underneath the arborized cells. Since only demecolcine treatment affected the formation of Lm-332- and Tn-C^L-immunoreactive plaques, we suggest that these proteins are vectorially secreted and directed from the Golgi apparatus underneath HCE cells via microtubules.

8.5.4 Adhesion of HCE cells to laminin-332, tenascin-C and laminin-332/tenascin-C (IV)

We have previously shown that HCE cells adhere to endogenous Lm-332 as well as to purified exogenous Lm-332. However, the cells did not adhere to Tn-C if cycloheximide was applied to inhibit endogenous protein synthesis. Our main interest was to elucidate the role of these proteins in early adhesion since both are deposited beneath the adhering HCE cells. The adhesion characteristics of HCE cells to Lm-332/Tn-C and Lm-332 coats were compared by quantitative cell adhesion experiments with or without cycloheximide. These experiments showed that HCE cells adhered similarly to both of these substrata, and

cycloheximide did not alter these results. Integrins α3 and β1 similarly functioned in the adhesion of HCE cells to both Lm-332/Tn-C and Lm-332. A hemidesmosomal component, integrin β4, which appears later during spreading of the HCE cell, did not function in the adhesion of HCE cells to these substrata.

Hemidesmosomes are known to mediate stable adhesion of keratinocytes (Borradori and Sonnenberg, 1996; Nguyen et al., 2000), and matricellular proteins, such as Tn-C, interfere with cell adhesion and induce an intermediate state of adhesion (Murphy-Ullrich, 2001). As corneal epithelial cells are required to migrate and cover the defective area rapidly, the assembly of hemidesmosomes would be unsuitable in early corneal wound healing. Immortalized gingival keratinocytes, on the other hand, produce and deposit Lm-332 similarly to HCE cells (Pakkala et al., 2002), but do not deposit Tn-C during early adhesion (Virtanen et al., unpublished data). Based on our results and earlier studies showing the role of Tn-C as a modulator of cell-ECM interactions, we suggest that Tn-C^L may prevent the assembly of hemidesmosomes when deposited together with Lm-332 in HCE cells. Pal-Ghosh et al. (2004) also hypothesized that the retention of Tn-C in the anterior stroma in corneal wounds could interfere with hemidesmosome reassembly. However, they suggested that Tn-C stops the activation of integrins α3ß1 and α6ß4 by inhibiting RhoGTP, which converts the migrating cells to a quiescent phenotype. This result is not supported by our study, and we suggest that Tn-C^L functions in the motility of HCE cells and cooperates with Lm-332 isoforms during corneal wound healing. Our results concerning the role of BM and ECM proteins in corneal wound healing are summarized in table 5.

Table 5. Production and function of basement membrane and extracellular matrix proteins in corneal wound healing

Production of BM and ECM proteins by HCE cells	Expression of BM and ECM proteins in in vitro wounds	Suggested functions for BM and ECM proteins in corneal wound healing
Lm-3′32	+	Epithelial cell spreading and migration
Lm-332	-	Epithelial cell adhesion
Tn-C^L	-	Prevention of the assembly of hemidesmosomes Epithelial cell migration
EDA-Fn	Not studied	Epithelial cell adhesion and migration Assembly of FAs
Onc-Fn	Not studied	Epithelial cell adhesion and migration? Assembly of FAs?

9. CONCLUSIONS AND FUTURE PROSPECTS

In vitro wound healing studies show that migrating HCE cells express the Lm α3′ chain along the wound edge and in supramarginal cells. In Addition, the Lm α3′ chain is found underneath adhering HCE cells, extending from the cell centre to the periphery, whereas the Lm α3 chain is deposited to restricted and centrally oriented plaques beneath HCE cells. We therefore suggest that Lm- 3′32 functions in spreading and migration of epithelial cells during corneal wound healing, while Lm-332 mediates the initial adhesion of these cells.

Our adhesion studies reveal that HCE cells do not adhere to Tn-C, although Tn-C is deposited together with Lm-332 underneath the adhering HCE cells. Lm-332 is known to function in the formation of hemidesmosomes via integrin α6β4. These structures mediate strong adhesion of basal corneal epithelial cells to the BM and disappear from the wound margins. Since integrin β4 does not function in the early adhesion of HCE cells and appears later during the spreading, we suggest that Tn-C may prevent the formation of hemidesmosomes when deposited with Lm-332. Hence, Tn-C could function in the migration of corneal epithelial cells during wound healing. However, in in vitro wound healing studies we could not find Tn-C in the migrating HCE cells, although this protein is present in cultured confluent cells. This discrepancy requires further consideration.

Integrin α3β1 and Lu mediated the adhesion of HCE cells to Lm-511, which promotes HCE cell spreading. During this adhesion both talin and Lu showed diffuse punctuate immunoreaction in HCE cells. On the other hand, when fibronectin was added to the adhesion substratum together with Lm-511, talin was located in FAs, while Lu remained diffusely distributed in HCE cells. FAs are known to play a role in both cell motility (Zaidel-Bar et al., 2003) and strong adhesion, which prevents cell migration (Murphy-Ullrich, 2001). We suggest that Lm-511 can favour the intermediate state of HCE cell adhesion by inducing cell spreading without inducing FAs. In addition, since Lm-511 does not prevent the formation of FAs, the cells can assemble these structures during migration.

HCE cells secreted EDA-Fn and Onc-Fn to the matrix and medium. Fibronectin serves as an adhesive substratum for HCE cells and induces integrins α5, αv, β1 and β6 to FAs. We found that integrin α5β1 mediated the adhesion of HCE cells to the RGD sequence of fibronectin. Although, integrin αvβ6 did not function alone in the adhesion of HCE cells to fibronectin, cell adhesion was inhibited nearly completely when both MAbs against integrin α5β1 and αvβ6 were applied together. The mechanism behind this phenomenon requires further examination. We found that Tn-C, which is secreted to the ECM, can modulate the adhesion of HCE cells to fibronectin. However, it did not affect FA formation induced by fibronectin. We conclude that also fibronectin, which is secreted to the provisional matrix during wound healing, cooperates with Tn-C during cell adhesion and migration.

The topical application of fibronectin ophthalmic solution has shown promising results in the treatment of persistent epithelial defects. Kimura et al. (2007) showed that the PHSRN peptide, a second cell-binding site of fibronectin, increased HCE cell motility in vitro. However, because fibronectin is isolated from blood and may be contaminated with infectious agents, it has not become a commercial product. Patients have instead been treated with autologous serum eye drops. Since these eye drops contain a large variety of growth factors, immunoglobulins and vitamins as well as fibronectin, it has proven to be a successful treatment for corneal epithelial wounds (Akyol-Salman, 2006; Geerling et al., 2004). Kabata et al. (1990) suggested that also topically applied vitronectin increases corneal wound healing. We propose that in the future the topical application of Lm-332 and vitronectin may have a place in the prevention of recurrent corneal erosion, as they mediate the adhesion of corneal epithelial cells. The combination of Lm-3′32, Tn-C^{L}, Lm-511 and fibronectin could be applied for the treatment of corneal epithelial wounds to accelerate cell migration.

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