Basement membrane heterogeneity

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BASEMENT MEMBRANE HETEROGENEITY

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PROEFSCHRIFT

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aan de Rijksuniversiteit Limburg te Maastricht,
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Jacobus Peter Marie Cleutjens
geboren te Oirsbeek in 1958

promotor:

prof.dr. F.T. Bosman

beoordelingscommissie:

prof.dr. P.J.C. van Breda Vriesman (voorzitter)

prof.dr. I. Damjanov

prof.dr. J. Drukker

prof.dr. J.P.M. Geraedts

prof.dr. Ph. J. Hoedemaeker

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Confetti

Confetti is alleen dan confetti als hij
(of zij?) méérkleurig is.
Het strooien van éénkleurige confetti
sorteert vrijwel geen effect,
't is de mengeling die het 'm doet
-c'est la vie!

Toon Hermans
Ik heb het leven lief (1981)

Aan mijn ouders
Aan Caroline

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List of abbreviations

BAPN	β -amino-propionitrile fumarate
BM	Basement membrane(s)
BSA	Bovine serum albumin
ECM	Extracellular matrix
EHS	Engelbreth-Holm-Swarm tumor
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal calf serum
GBM	Glomerular basement membrane
HSPG	Heparan sulphate proteoglycan
kD	KiloDalton
MA	monoclonal antibod(y)ies
Mw	Molecular weight
NC1	non-collagenous domain 1 of type IV collagen
NEM	N-ethylmaleimide
PA	polyclonal antiser(um)a
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
Pen	Penicillin
RIA	Radioimmunoassay
RT	Room temperature
Strep	Streptomycin

Chapter 1

General introduction

1.1 Introduction

The basement membrane (BM) is a special component of the extracellular matrix (ECM), which is deposited as a sheet-like structure in close vicinity to the adherent cell. The BM may be deposited in a polarized fashion at the basal side of some cells (epithelial and endothelial cells) or may completely surround individual cells such as adipocytes, myocytes, and Schwann cells (1). Several important biological functions are ascribed to BM. They are responsible for tissue compartmentalization in maintaining tissue architecture. They provide adequate anchorage for adjacent cells, which thus maintain their polarized and differentiated state. They control cell migration and invasion and they can act as selective filtration barriers, as in the capillaries of the renal glomerulus (2-5).

1.2 BM biosynthesis and turnover

The extracellular matrix, including the BM was regarded until recently as a passive and inert structural framework for the epithelial and mesenchymal cellular components of a tissue (6). Fully assembled BM are indeed relatively stable. Turnover rates from 40 days in the colon to more than 2 years in skin were estimated on the basis of the rate of silver stain loss (7). *In vivo* labeling of glomerular BM with anti-laminin antibodies showed that the antibodies remained bound for several months (8). Metabolic labeling with radioactive amino acids showed that collagenous components have a slower turnover rate than non-collagenous components (9). Faster turnover rates have been found during morphogenesis, as in tooth development, and pathological conditions, such as neoplasia.

It is generally assumed that epithelial BM are at least partly produced and deposited by the epithelial cells (10, 11). *In vitro*, epithelial cells are usually capable to produce BM components and secrete these into the culture medium (12-15).

Mesenchymal cells such as Schwann cells, muscle cells and fat cells are also capable of BM production (2). These cells produce and deposit the extracellular matrix components into a normal BM *in vivo*. *In vitro*, Schwann cells (16) and muscle cells (17) have been shown to produce and deposit BM-like structures. Evidence exists that mesenchymal cells contribute to the BM formation. Cocultivation of mouse muscle fibroblasts and quail skeletal myoblasts results in deposition of a BM of mouse origin (18, 19), and combination of chick mesenchyme and rat endoderm results in the formation of a BM of chick origin (20). Xenotransplantation of human tumor cells into athymic animals resulted in the formation of BM-like structures with components of both host and tumor cell origin (21, 22). These results indicate that BM are deposited as a result of cellular interactions: between epithelial and mesenchymal cells for epithelial BM and between different mesenchymal cells for mesenchymal BM (myocytes, adipocytes, Schwann cells and endothelial cells).

1.3 General structure of the BM

1.3.1 Morphology

Electron microscopically most BM appear as a three layered structure (2, 5, 23) as illustrated in Fig 1. The lamina lucida, also called lamina rara, is an electron lucent layer adjacent to the plasma membrane of the adherent cell. In the lamina lucida very delicate anchoring filaments occur, which traverse this layer perpendicular to the plasma membrane (24). The lamina densa is an electron dense layer, situated at the stromal side of the lamina lucida, and at high magnification appears as a network of cords (25). Finally, underlying the lamina densa the subbasal lamina or lamina fibroreticularis occurs, which contains fibrils anchoring the BM onto the underlying stroma. These consist of anchoring fibrils, elastic microfibrils and fine interstitial fibrils, which probably represent reticulin (25). Epithelial cells are attached to the BM by hemidesmosomes.

The hemidesmosomes contain an intracellular attachment plaque, which is in contact with the plasma membrane, intracytoplasmic actin and keratin tonofilaments, and an extracellular subbasal dense plate located in the BM.

Figure 1. Electronmicrograph of peripheral (conjunctival) human corneal epithelial basement membrane. E = epithelium; LL = lamina lucida; LD = lamina densa; AF = anchoring fibers. Scale bar = 0.1 μm .

Anchoring filaments are more numerous in the hemidesmosomal region than in the region between hemidesmosomes. Evidence exists that anchoring fibrils occur predominantly at the hemidesmosomal sites (25-27). Consequently the anchoring of epithelial cells is mediated by a complex network of intracytoplasmic filaments, hemidesmosomes, BM components and anchoring fibrils. Another mechanism of cell-matrix attachment is mediated by specific receptors for extracellular matrix components on the cell surface (28, 29). The best characterized ligand is the arginine-glycine-aspartic acid (RGD) sequence (30, 31), which is recognized by receptors of the integrin family (32). Other attachment receptors are the laminin receptors (33-35), and anchorin, a collagen receptor (36). It is likely, however, that attachment of cells to the ECM is also mediated by specific receptors for other ECM components (37, 38) as schematically outlined in Fig 2.

1.3.2 Chemical composition

Unlike most other biological membranes, such as plasma and organelle membranes, BM contain little or no lipid (39). The BM constituents are highly insoluble because of many covalent and non-covalent interactions. Consequently the separate components are difficult to isolate, which has hampered their structural characterization in man.

The study of BM biochemistry has been facilitated through the availability of BM producing neoplasms, which can be transplanted into lathyrotic animals. These models, including the mouse Engelbreth-Holm-Swarm (EHS) tumor (40) and the rat yolk sac carcinoma (41, 42), have allowed the recognition and chemical characterization of the most important BM components. The major constituents, including type IV collagen, laminin, heparan sulphate proteoglycan and entactin/nidogen have been found in nearly all BM. It is likely, however, that many more BM components exist. We will first discuss the characteristics of the BM components found in all BM and will follow with a discussion of BM components with a limited tissue distribution.

1.3.2.1 Components of all BM

*** Type IV collagen**

Type IV collagen is the structural component of the BM and has a molecular weight of about 550-600 kD (2, 39). Type IV collagen consists of two distinct peptide chains, $\alpha_1(\text{IV})$ and $\alpha_2(\text{IV})$, assembled into a triple helical structure. Recently two new peptide chains were found: an $\alpha_3(\text{IV})$ and (tentatively) $\alpha_4(\text{IV})$ -chain (43). Different homo- and heterotrimers can be formed, which might show tissue or cell specific distribution. This area has, however, not been fully explored as yet. Type IV collagen differs from the interstitial collagens (type I, II and III) in its multidomain structure (44).

Furthermore the triplet sequence Gly-X-Y shows frequent interruptions in which X or Y often is proline or hydroxyproline (45). The type IV collagen molecule consists of a major triple helix which is connected at its amino-terminal domain with three other amino-termi-

Figure 2. Schematic drawing of the basement membrane and its attachment properties to epithelial cells and underlying stroma. 1 = epithelium, 2 = lamina lucida, 3 = lamina densa, 4 = lamina fibroreticularis, 5 = anchoring fibers, 6 = anchoring plaques, 7 = extracellular matrix receptors, 8 = hemidesmosomes (a = intracellular attachment plaque, b = anchoring filaments, c = subbasal dense plate).

nal domains, partly by covalent interactions, which together form the 7S-domain (Mw 25 kD) (46-48). The globular carboxy-terminal domain is another cross-linking site, participating in the formation of a three dimensional matrix. Here, two type IV collagen molecules are connected with each other, partly by disulfide bonds and partly by covalent cross-linking, to form the non-collagenous (NC1) domain (Mw 160-170 kD) (49-51). Six globular end-regions assemble in a hexameric configuration. Interactions along the type IV collagen molecule can also initiate lateral assembly (52). Within the triple helix, another non-collagenous segment can be found which has been designated as the NC2-domain (Mw 10 kD). This segment contains triple- and non-triple helical sequences. The NC2 domain acts presumably as a flexible hinge (39). Type IV collagen is insoluble in tissues due to the formation of large networks which are stabilized by disulfide bridges and nonreducible cross-links (2, 39, 5).

*** Laminin**

Laminin is a 900 kD noncollagenous glycoprotein, composed of several fragments that form a asymmetric cross-shaped structure. By immuno-electron microscopy laminin can be detected throughout the entire BM but with a higher concentration in the lamina lucida (2, 53). Some investigators found laminin exclusively in the lamina lucida (54-56). By rotary shadowing electron microscopy, laminin appears as a cruciform molecule with one long arm (75 nm), which contains a large globular end-domain, and three short arms (35 nm), each with a terminal globular end-domain and another globular domain in the centre of the molecule. These arms are interconnected by disulfide bonds. Globular domains at the end of the short arms bind to type IV collagen (57). The central part of the laminin molecule binds to cell surfaces via plasma membrane receptors (58, 59). Binding to heparan sulphate proteoglycan is mediated by specific domains on the long arm (60).

Laminin has several biological functions. It plays a role in cell attachment, cell spreading, mitogenesis, neurite outgrowth, morphogenesis and cell movement (3). Furthermore laminin is an important structural component of the BM. Because it binds to type IV collagen, heparan sulphate proteoglycan and entactin (37). It is thought that laminin acts as a major linker holding together the entire BM and thus determine its architectural properties.

*** Heparan sulphate proteoglycan**

Heparan sulphate proteoglycan (HSPG) consists of a core protein and glycosaminoglycan side chains (61, 62). As much as 50 to 80% of the entire molecule consists of glycosaminoglycans (62). The core protein of HSPG from the bovine glomerular BM (MW 128 kD) has been identified and appears to be unique for BM HSPG (62). HSPG occurs in the BM predominantly in the lamina lucida where it forms clustered aggregates (63). Other investigators have detected HSPG also in the lamina densa (64). HSPG probably plays a rôle in the attachment of cells to BM and, through its anionic properties, in the regulation of glomerular filtration (63, 65). In addition to HSPG, proteoglycans with chondroitin- and dermatan sulphate side chains have been found in BM (66).

*** Entactin/nidogen**

Entactin is a major BM component, with a molecular weight of 150 kD. It is a sulfated glycoprotein and was first discovered by Carlin et al. (67). It was rediscovered by Timpl et al. (68) and named nidogen. The protein is highly susceptible to proteolysis (68, 69). Its self aggregating tendency and affinity for laminin, fibronectin and type IV collagen (70) strongly suggest that the molecule has a bridging function within the BM. Entactin has no affinity for heparan sulphate proteoglycan (71). One entactin molecule forms a complex with laminin by attachment to one of the short arms of laminin, near to the center of the cross (72). Recently the complete amino acid sequence has been derived from sequencing

cDNA clones (73). It was shown that entactin has homologous sequences with epidermal growth factor and low density lipoprotein receptor.

*** LDA-1**

A newly discovered BM component but still incompletely characterized is the antigen detected by the LDA-1 monoclonal antibody. By immunohistochemistry the LDA-1 epitope is localized in the lamina densa of all BM. The epitope is collagenase insensitive and protease sensitive, suggesting that it is of noncollagenous nature (74).

1.3.2.2 Components with a limited tissue distribution

The previously mentioned components are present in almost all BM. Many other substances have been found in BM but with a limited distribution (only in some BM) and/or not restricted to the BM (occurring also in interstitial stroma).

*** Anchoring fibers**

Anchoring fibers occur in the lamina fibroreticularis, and anchor the BM to the underlying mesenchyme. Anchoring fibers are found in BM of tissues of ectodermal origin, such as BM of the epidermis and the epidermal adnexa. Type VII collagen is the major structural component of anchoring fibers (75, 76). Anchoring fibers form horseshoe shaped structures, originating from and inserting into the lamina densa, or extending perpendicularly into the upper regions of the mesenchyme (24, 77). In the skin anchoring fibers are often associated with amorphous dermal (anchoring) plaques, which are not BM undulations. These plaques contain type IV and VII collagen (78). Antibodies specific for anchoring fibers (AF-1 and AF-2, LH 7:2, KF-1) have been generated (79-81). Monoclonal antibodies to type VII collagen have also been made (76). These antibodies bind exclusively to the dermal/epidermal junction, sometimes including the junctions of the epidermal adnexa and the epithelial BM in oesophagus and trachea.

*** Chondroitin sulphate proteoglycan**

Chondroitin sulphate proteoglycan has been observed in small amounts in the dermal/epidermal junction of the human skin BM (66, 82).

*** Type V collagen**

Type V collagen has been described as a BM component (2, 83), although it is now generally regarded as an interstitial rather than a BM collagen. Type V collagen is not easily detected in the interstitium because of its intrafibrillar organization at the interior of type I and/or type III collagen fibers (84-86).

* Fibronectin

Fibronectin is a BM associated molecule rather than a true BM component (2). It is not confined to a single BM layer (87), it occurs in variable amounts in different BM and the BM of the same kind differ with regards to their content of fibronectin. Fibronectin is also found in the interstitium and in the circulation (2, 88).

1.3.3 BM heterogeneity

So far we have mainly discussed the characteristics common to all or most BM. It has become increasingly clear that BM in different tissues show characteristic variations in structure and composition. Furthermore under pathological conditions BM may show additional characteristic alterations contributing even more to the general BM heterogeneity.

1.3.3.1 Normal variations

* Morphology

By electron microscopy, the BM consists of at least two layers: the lamina lucida and lamina densa. The lamina fibroreticularis, containing the anchoring fibers, is present only in the BM of the epidermis and epidermal adnexa, glandular epithelium of breast and prostate, squamous epithelium of the tongue, oesophagus, and vagina, and ciliated epithelia of the trachea and bronchus (76).

The glomerular BM is a unique structure since it consists of a fused endothelial and epithelial BM. Thus it appears as a trilaminar layer with a duplicated lamina densa, sandwiched between an epithelial lamina rara externa and an endothelial lamina rara interna (2). The only other BM of this appearance is the pulmonary alveolar BM, formed also from fused endothelial and epithelial BM.

Ultrastructurally the lamina densa varies somewhat in thickness: it is often thicker where hemidesmosomes occur (27). This observation suggests that components of the subbasal dense plate of the hemidesmosome might be intertwined with the BM components.

Pericellular BM may appear by electron microscopy as a continuous or discontinuous layer. Thus e.g. in myocytes, it appears continuous whereas myofibroblasts are surrounded by an interrupted BM. Patches of BM components can be visualized as dense material, surrounding the myofibroblasts and containing at least type IV collagen, laminin and heparan sulphate proteoglycan (89-91). Fenestrations of the BM have been observed in rat and monkey intestinal villi, with a higher number of fenestrations above the Peyer's patches (92, 93). These holes in the BM might represent temporary tracks created by lymphocytes migrating from the lamina propria to the epithelium. In the rat liver, the hepatic extracellular space (space of Disse) has no linear BM. Small patches of type IV collagen without laminin can be observed (94, 95). The discontinuous BM facilitates the rapid exchange of

macromolecules between plasma and hepatocyte. However, in cirrhosis the BM becomes linear and type IV collagen and laminin are codistributed, transforming most of the hepatic sinusoids into capillaries.

*** Composition**

Although type IV collagen has been described as a structural component of all BM, type IV collagen does not appear to be present in the central corneal basement membrane (96-99), suggesting that the central cornea has a structurally different BM. Furthermore normal BM components can show variations in the molecular conformation. Different immunohistochemical staining patterns for laminin in different tissues have been demonstrated using monoclonal and polyclonal antibodies to different epitopes of laminin (100, 101). This is also suggested by experiments with protease treatment of tissue sections which have shown that tissues have different protease susceptibility, pointing towards qualitative differences in the BM components (102).

BM heterogeneity is reflected also in the concentration of certain BM components, detected biochemically (2). In lens capsule 90% of the total dry weight is type IV collagen, whereas the glomerular BM contains 45% and the Reichert's membrane only 25% type IV collagen (23).

Because BM are highly cross-linked structures, their components cannot be isolated easily and extensive enzyme treatment is most often needed for biochemical analysis. Through this treatment BM components may be lost or may disintegrate and components which occur in very low amounts could remain undetected (103). Therefore, new approaches have to be derived such as e.g. production of monoclonal antibodies to native BM components. This approach may lead to the detection of hitherto unknown BM constituents (103-106), although the characterization of new components could pose new and often unsurroundable obstacles.

1.3.3.2 Pathologic variation

Under pathologic circumstances BM may show alterations in morphology and composition exceeding the variation encountered in normal tissues. Investigations concerning these alterations have yielded important information with regard to the structure and composition of the normal BM but also shed light on the role of the BM in the pathogenesis of various diseases.

*** Morphology**

In various pathological conditions BM morphology shows characteristic alterations. Thus in diabetic microangiopathy capillary BM are thickened. In contrast, in the thin BM glomerulopathy and sometimes in Alport's syndrome, the glomerular BM is abnormally thin. Finally, in neoplasia BM may become discontinuous and irregularly thickened.

Vascular BM thickening is a common feature in diabetes mellitus. This could be due to a decreased content of heparan sulphate proteoglycan in these patients, in combination with an increase in the concentration of type IV collagen and laminin. BM thickening might occur as a compensatory phenomenon in the glomerular BM in order to restore the altered BM porosity and malfunction in filtration due to the loss of heparan sulphate proteoglycan (2, 107, 108). In lipid proteinosis the BM appears multilamellar or reduplicated (123, 124).

In contrast to BM thickening, early stages of Alport's disease are marked by diffuse thinning and splitting of the glomerular BM takes place (109). In thin BM nephropathy, BM thinning was observed with quantitative electron microscopy in glomeruli from a subset of patients with persistent microscopic erythrocyturia (110).

Tumor invasion can be defined as extension of neoplastic cells beyond the natural borders of the tissue or cell type from which they are derived. As a result of this active process of invasive growth the neoplastic cells occur in tissue compartments where they do not belong. The existence of specific mechanisms for degrading BM during tumor invasion was strongly indicated by the characterization of a metalloproteinase which specifically cleaves type IV collagen (111). Other enzymes, such as cathepsins, elastase and other proteases are frequently associated with early invasion (112). Changes in biosynthesis or metabolism of BM components, which can lead to BM thickening or BM loss also affect invasiveness of tumor cells (38, 39, 113, 114).

For epithelial tissues BM are the natural borders, separating them from the connective tissue stroma. Consequently, in invasive carcinoma, the malignant epithelial cells dissolve the epithelial BM and migrate into the surrounding mesenchymal stroma. Thus it is understandable that the BM adjacent to carcinoma cells are usually discontinuous (2, 5, 38, 115, 116). Immunohistochemical staining of BM has proved to be helpful to differentiate between benign or premalignant and malignant lesions: discontinuities are usually limited to malignant lesions (38, 117, 118). However, exceptions have been found to this rule: BM discontinuities do occur in benign neoplasms and conversely, intact BM have been reported in malignant tumors (119, 120). In adenoid cystic carcinoma (121, 122) the BM becomes a multilamellar or reduplicated structure due to excessive production and deposition of BM components.

*** Composition**

The chemical composition of the BM in pathological conditions has not been studied extensively. Rather unique components of the BM do, however, play an important role in the pathogenesis of immune-mediated disorders, such as the Goodpasture syndrome and the group of bullous dermatoses. In all these conditions the composition of the BM changes due to the deposition of immunoglobulins. An interesting aspect of these disorders is that identification of the target antigens of these disorders may alter recognition and further characterization of new BM components.

* Goodpasture syndrome

Goodpasture syndrome (125), is characterized by pulmonary hemorrhages preceding the rapidly progressive glomerulonephritis, with which it is frequently associated. This disease is characterized by autoantibodies to BM which form diffuse linear deposits along the pulmonary and cross-react with the glomerular BM. In diffuse crescentic glomerular nephritis caused by anti-glomerular BM antibodies, pulmonary hemorrhages are caused by pulmonary venous hypertension secondary to the renal insufficiency. Although the epitopes of the Goodpasture autoantibodies occur in all BM, the primary lesions are in the lungs and kidneys. Recent studies have shown that the Goodpasture antigen is sequestered inside the hexameric structure of the NC1 domain of type IV collagen (50). Immunoblotting experiments have shown that autoantibodies from Goodpasture patients react with the monomeric M2* and dimeric D2* bands of collagenase released hexamers. These epitopes are localized in the $\alpha 3(\text{IV})$ chain of type IV collagen (43).

* Bullous pemphigoid

Bullous pemphigoid is a dermal disease with formation of subepidermal bullae within the lamina lucida, between the plasma membrane of the basal cells and the lamina densa. Furthermore this disease is characterized by *in vivo* bound immunoreactants along the epidermal-dermal junction. By electron microscopy these immune complexes appear locally in the lamina lucida. The bullous pemphigoid antigen has been identified as a disulfide-bonded 220 kD polypeptide (126). The bullous pemphigoid antigen is a component of hemidesmosomes (127). In 70 to 80% of these patients autoantibodies of IgG-class reactive with epidermal BM can be demonstrated (64).

* Cicatricial pemphigoid

Cicatricial pemphigoid or benign mucous membrane pemphigoid is a bullous disease leading to scarring in oral and conjunctival lesions (128). Circulating autoantibodies against BM can be demonstrated in 10-40% of the patients. Frequently linear immune complexes are present in the lamina lucida of the epidermal BM. It is not unlikely that bullous pemphigoid and cicatricial pemphigoid are variants of the same disease (64).

* Epidermolysis bullosa

Epidermolysis bullosa comprises a group of rare genetic disorders which have in common the formation of blisters occurring after minor mechanical injury. It presents in a variety of forms. The so called dystrophic epidermolysis bullosa is a subset of this disorder characterised by dermal tissue cleavage below the BM. Tissue bound immunoreactants against BM are always present and can be demonstrated by immuno-electron microscopy in the lamina densa and below the lamina densa, in the lamina fibroreticularis. Circulating auto-

antibodies are present in 40% of the patients (64). The upper dermis contains decreased amounts of interstitial collagens and increased amounts of elastic fibers. By electron microscopy, the cleavage appears to be beneath the BM. This disease is related to rudimentary anchoring fibrils, which also seem to be reduced in number. The epidermolysis bullosa acquisita antigen has been chemically recognized as a 290 kD protein which forms two subunits of 145 kD after reduction (129).

1.4 Aim of the study

From the literature reviewed in the previous paragraphs it can be concluded that BM of different cells or tissues vary and that these differences occur related to the specialized functions of the cells or tissues, or due to pathologic processes.

In this thesis some aspects of the chemical and structural heterogeneity of the BM in normal and pathological situations will be investigated, primarily with monoclonal antibodies. In chapter 2 the production of monoclonal antibodies, raised against native, non-enzymatic treated BM from amnion or glomeruli is described, as well as the immunohistochemical characterization of these monoclonal antibodies and the heterogeneous nature of their reactants recognized in the BM. In chapter 3 the molecular configuration identified by these monoclonal antibodies is investigated. The monoclonal antibodies are reactive with different epitopes of the globular carboxy-terminal end region of type IV collagen. Although some monoclonal antibodies do not react with mesenchymal BM by immunohistochemistry, the epitope can be detected after enzymatic treatment of the tissue followed by electrophoresis and immunoblotting, indicating that the type IV collagen structure is dependent upon the tissue in which it occurs.

In chapter 4 and 5 morphological and chemical heterogeneity of BM in normal organs and cells is discussed. The issue of type IV collagen being an essential structural component of all BM is discussed in chapter 4. The immunohistochemical heterogeneity of the central epithelial corneal BM, using antibodies to type IV collagen, is reported and compared with the staining pattern of other BM components. Furthermore the ultrastructural heterogeneity of the central versus peripheral corneal epithelial BM is correlated with the immunohistochemical absence of type IV collagen. The question of the continuity of the pericellular BM in normal tissue is addressed in chapter 5. The stromal cells of the umbilical cord were investigated ultrastructurally as cells enveloped by discontinuous BM-like deposits. Furthermore these cells were characterized by immunohistochemistry using antibodies to cytoskeletal and BM components. Their electron microscopical features were compared with those of myofibroblasts found in the desmoplastic response to neoplasia and in healing wounds. This cell type is surrounded by discontinuous patches of BM components, both *in vivo* and *in vitro* explants. Production of type IV collagen *in vitro* is demonstrated by northern analysis and *in situ* hybridization using a type IV collagen cDNA probe and immunohistochemically on cells grown on glass slides and immunoblots of tissue culture supernatants.

Heterogeneity of BM in pathological conditions is discussed in chapter 6 and 7. One of the most intriguing questions is the origin of BM in epithelial neoplasms. The contribution of mesenchymal cells to the production and deposition of BM components into an epithelial/mesenchymal BM, as investigated in tumor cell lines xenotransplanted into athymic animals, is reported in chapter 6. The origin of type IV collagen was investigated by immunohistochemistry using antibodies to rat/murine host and human specific epitopes of type IV collagen. These data were compared with the detection of mRNA, coding for type IV collagen, via in situ hybridization with a cross-species reactive type IV collagen cDNA probe. As shown in this chapter, the epithelial BM in these xenotransplanted tumor cells is of epithelial or mesenchymal origin or both. Stromal factors regulating the deposition of BM were investigated in chapter 7. Finally, in chapter 8, the results of these studies concerning BM heterogeneity, production, origin and deposition are discussed against the background of recent literature. The prospects for future studies are briefly outlined.

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Chapter 2

Monoclonal antibodies to native basement membranes reveal heterogeneous immunoreactivity patterns*

*Cleutjens JPM, Havenith MG, Vallinga M, Beek M, Bosman FT.
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2.1 Introduction

Until now it has been described that all basement membranes (BM) contain type IV collagen, laminin, heparan sulphate proteoglycan and entactin (1). Type IV collagen constitutes the basic component of the BM to which the other components are attached (2). Although the basic structure of all BM is identical, it is likely that BM in different anatomic sites have unique components, which might be related to a specific local function. Unique tissue specific BM characteristics have been recognized in some clinical syndromes. Thus bullous pemphigoid, is characterized by autoantibodies against the bullous pemphigoid antigen in the epidermal BM (3) and the Goodpasture syndrome, characterized by pulmonary hemorrhages preceding the rapidly progressive glomerulonephritis, with which it is frequently associated. This disease is characterized by autoantibodies to BM which form diffuse linear deposits along the pulmonary and cross-react with the glomerular BM (32). Although the epitopes of the Goodpasture autoantibodies can be found in all basement membranes after appropriate pretreatment, the disease affects mainly the lungs and kidneys (4).

Additional findings suggesting BM heterogeneity derive, from immunohistochemical (8) and lectin histochemical studies (5, 6). Several investigators have reported heterogeneous BM staining patterns with monoclonal antibodies (MA) against laminin (7, 8) or against type VII collagen (9, 10). Others have induced MA against amnion BM (11, 12) or human glomerular BM (13), which display heterogeneous BM staining patterns. Heterogeneous BM reactivity with MA generated against human fetal membranes and human glomerular basement membranes will be reported in this chapter.

2.2 Materials and methods

2.2.1 Immunogens

Human amnion was separated from chorion and the amniotic BM was isolated by chemical and mechanical treatment as described by Liotta et al. (14). Briefly, amnion was washed one hour in distilled water with 2 mM N-ethyl maleimide (NEM), extracted one hour in distilled water with 1 M NaCl, 20 mM EDTA, 2 mM NEM and finally one hour in distilled water with 4% de-oxycholate. After each step the membranes were mechanically treated with a rubber policeman to remove amniotic epithelial cells and most of the interstitial stroma. Types IV and V collagen were isolated from the human placenta by pepsin digestion and limited salt precipitation, followed by column chromatography as described earlier (15). Human glomerular basement membrane extracts were kindly provided by Dr. B. van den Heuvel (Nijmegen). These had been prepared according to the procedure described by Langeveld et al. (16). In short human kidney cortex was sieved sequentially on several mesh sizes. Glomeruli were obtained at mesh sizes between 90 and 125 μm . The glomeruli were lysed in 0.1% NaN_3 and 1 mM EDTA, centrifuged and subsequently incu-

bated 5 h with 4% sodium de-oxycholate, treated 2.5 h with deoxyribonuclease (59 Kunitz units/ml), washed with distilled water and lyophilized.

2.2.2 Immunization and monoclonal antibody production

Balb/c mice were immunized by subcutaneous implantation of stripped amniotic membranes and boosted with amnion supplemented with types IV and V collagen and an 8 M urea extract from amniotic membranes (17), as extensively described in chapter 9.1. Immune spleen cells were fused with Sp2/0 cells (18) and resulting hybridomas were selected by immunohistochemistry on placental membranes, following standard techniques with minor modifications as outlined in chapter 9.2.

2.2.3 Antibodies

Antibodies used in this study were the monoclonal antibodies generated as described above and furthermore monoclonal and polyclonal anti-type IV collagen antibodies (15), monoclonal and polyclonal anti-laminin antibodies (7), a monoclonal anti-type VII collagen antibody (9) and a monoclonal anti-heparan sulphate proteoglycan antibody. The specificities of these antibodies are outlined in chapter 9.4.

2.2.4 Tissues

Samples of various human tissues, obtained at autopsy or from surgical specimens, were snap frozen in isopentane, quenched in liquid nitrogen. Frozen tissues were cryostat sectioned at 4 μ m. Immunocytochemistry was performed as described for hybridoma testing. In addition, for immunofluorescence, rabbit anti-mouse IgG conjugated with fluoresceine isothiocyanate (Dakopatts) was used as a second step.

2.2.5 Digestion of frozen sections

Unfixed frozen sections were incubated during 30 minutes at room temperature (RT) or 37 °C with various concentrations of the following enzymes: trypsin (Boehringer, Mannheim, FRG), 0.05-0.5% pepsin (Boehringer, Mannheim, FRG) in 0.1 N HCl (30 min, RT), 0.01-0.10% pronase (Boehringer, Mannheim, FRG) in 50 mM Tris-HCl, pH 8.0, (30 min, RT), collagenase (*Clostridium histolyticum*; Sigma, St. Louis, USA) and 0.05-0.1% hyaluronidase (Cooper Biomedical) in 0.1 M sodium phosphate buffer (0.15 M NaCl, pH 4.5, 30 min, RT). Furthermore the sections were eluted with 6 M guanidinium hydrochloride (GuHCl), or 8 M urea in 50 mM sodium acetate, 50 mM EDTA, pH 6.5, 30 min, RT, or treated with 0.1-0.5 M HAc, 30 min, RT. These procedures were chosen to grossly determine the nature of the detected antigen (enzymes) or to unmask possible hidden epitopes (tissue extraction with GuHCl or urea, tissue swelling with HAc).

2.2.6 Chemical cleavage of the epidermal basement membrane

Fragments of human abdominal skin, approximately 4 cm², obtained at autopsy, were incubated during 24 hours at 4 °C with 1 M NaCl as described by Fine et al. (19). After incubation, the epithelial cell layer could be removed by pulling the epidermis from the dermis with a forceps. The plane of cleavage appeared to be within the basement membrane. After separation, the epidermal and dermal layers were frozen and sectioned. The immunoreactivity of the different layers with the various antibodies was investigated to localize the epitopes of the MA into the different layers of the BM.

2.3 Results

2.3.1 Hybridomas obtained

Testing of hybridoma culture supernatants on frozen sections of intact placental membranes resulted in twenty BM immunoreactive hybridomas (e.g. BM restricted and/or amniotic epithelial cell reactive and/or interstitial stroma reactive).

The pattern of reactivity on human fetal membranes of clones 1052 (IgG1), 1053 (IgG1), and 1065 (IgG2b) with a predominantly BM restricted staining pattern, is summarized in Table 1. Of the species tested (including man, rat, rabbit, swine, and dog) these MA reacted only with human tissues. All MA stained the amniotic BM, whereas none were reactive with placental BM. Clone 1065 showed weaker immunoreactivity of the BM in combination with cytoplasmatic reactivity of amnion epithelium, some chorionic epithelial cells and trophoblastic cells of the villi. All MA stained the chorionic BM, but MA 1052 and 1053 showed a much more intense immunoreactivity than 1065. All MA showed pericellular immunoreactivity of chorionic epithelial cells. Reactivity was found only with unfixed cryostat sections. Even mild fixation (3 min, RT, 2-4% phosphate buffered paraformaldehyde) destroyed the immunoreactivity.

The MA 1042 and 1043 directed against pepsin cleaved type IV collagen (15) and clone 1087 (IgG2b) reacted with all basement membranes in the fetal membranes and placental tissue.

2.3.2 Reactivity on human tissues

Human specific anti-type IV collagen reactive MA (clones 1042 and 1043) stained nearly all BM. The immunoreactivity of MA 1087, obtained after immunization with human glomerular basement membrane extracts, showed the same pattern of immunoreactivity as MA 1042 and 1043. Only the central epithelial basement membrane of the cornea did not show immunoreactivity with MA 1042, 1043 and 1087, whereas antibodies to laminin and heparan sulphate proteoglycan showed uninterrupted staining of the corneal epithelial basement membrane (20).

Table 1. Immunoreactivity of the monoclonal antibodies with fetal membranes and placenta

Monoclonal Antibody	amnion		chorion cytotrophoblast			placenta	
	BM	ic	BM	pc	ic	BM villus	trophoblast
1042	++	—	+	+	—	+	—
1043	++	—	+	+	—	+	—
1087	++	—	+	+	—	+	—
1052	++	—	++	+	—	—	—
1053	++	—	++	+	—	—	—
1065	+	+	+	+	+/-	—	+/-

BM = basement membrane; ic = intracytoplasmic; pc = pericellular; ++ = strong positive; + = positive; — = negative; +/- = moderate immunoreactivity

MA 1087 displayed less intense immunoreactivity than MA 1042 and 1043, which showed staining intensity comparable to that with antibodies to laminin and heparan sulphate proteoglycan. This heterogeneity in staining intensity was most clearly seen in the human liver. MA 1042 and 1043 showed intense immunoreactivity with the hepatocyte or sinusoidal basement membrane, whereas MA 1087 and antibodies to laminin and heparan sulphate proteoglycan showed very weak immunoreactivity.

The reactivity of the other MA is summarized in Table 2 and Fig 1. MA 1052 and 1053 homogeneously stained BM of epidermis and epidermal adnexa, such as sebaceous and sweat glands and hair follicles. Furthermore BM of glandular epithelium of breast and prostate, squamous epithelium of the tongue, oropharynx, oesophagus, and vagina, and ciliated epithelia of the trachea and bronchus as well as alveolar BM in the lung were stained. In the lung no reactivity occurred with the endothelial basement membrane.

In the gastrointestinal tract epithelial BM of the superficial crypt (colon) or villous (small intestine) structures was observed, whereas deeper in the mucosa BM reactivity was absent. No reactivity was obtained with renal or vascular BM or with BM of fat-, muscle-, or Schwann cells.

MA 1065 in contrast to the staining pattern of MA 1052, 1052 and 1087 did not react with BM in the lung and the gastrointestinal tract. However, the tracheal epithelial BM and the BM of the oesophageal mucosa reacted with this MA. Furthermore MA 1065 showed, in addition to basement membrane staining, strong pericellular immunoreactivity with the basal cells of the epidermis. MA 1065 did not react with renal or vascular BM or with BM of fat-, muscle-, or Schwann cells.

Figure 1. Indirect immunofluorescence staining of cryostat sections of human skin (A,B,C) and colon (D,E,F). A and D are stained with anti-type IV collagen antibody (clone 1042). Note continuous labeling of all BM. B and E are stained with clone 1052. Note staining of the epithelial BM only. In colon (E) staining is most intense at the luminal border. C and F are stained with clone 1065. Only epidermal BM membrane is stained. Furthermore the basal cell layer shows pericellular labeling. Scale bar = 10 μ m.

Table 2. Immunoreactivity of the monoclonal antibodies with BM of human tissues

Tissues	monoclonal antibody				
	1042/ 1043	1087	1052/ 1053	1065	4E10/ HSPG
Skin: epidermis	+	+	+	+	+
adnexa	+	+	+	+	+
Cornea	—	—	+	+	+
Breast	+	+	+	+	+
Tongue	+	+	+	+	+
Oropharynx	+	+	+	+	+
Vagina	+	+	+	+	+
Oesophagus	+	+	+	+	+
Trachea	+	+	+	+	+
Bronchus	+	+	+	—	+
Stomach	+	+	+	—	+
Small intestine	+	+	+	—	+
Colon	+	+	+	—	+
Lung	+	+	+	—	+
Liver	++	+/-	—	—	+/-
Kidney	++	++	—	—	+

+ = positive; — = negative; +/- = weak immunoreactivity; sup = superficial epithelium;
pc = pericellular immunoreactivity

2.3.3 Chemical cleavage of the epidermal BM

The sodium chloride split epidermal BM was frozen, sectioned and stained with the available antibodies (Table 3 and Fig 2). MA 1065 showed very strong pericellular staining of basal epidermal cells and adherent basement membrane components but no staining of BM adherent to the dermis. Laminin was predominantly found in the epidermal part but also faintly in the BM layer adhering to the dermis. Antibodies to type IV collagen, however, predominantly stained the BM-layer adhering to the dermis, whereas the epidermal adherent BM components showed only faint immunoreactivity. The MA 1052, 1053 and antibodies to type VII collagen reacted exclusively with the BM segment adherent to the dermis, indicating that the epitope is localized in the (sub)lamina densa.

Figure 2. Immunoperoxidase staining of cryostat sections from NaCl cleaved human skin. Epidermal face (A, C, E). Dermal face (B, D, F). A and B are stained with MA 1042. C and D are stained with MA 1052. E and F are stained with MA 1065. Scale bar = 10 μ m.

Table 3. Immunoreactivity of monoclonal antibodies after chemical splitting of the epidermal basement membrane

antibodies	type	immunoreactivity	
		epithelial layer	stromal layer
1042/1043	ma	+/-	++
1087	ma	+/-	++
4E10	ma	+	+/-
1052/1053	ma	-	++
1065	ma	++ (pc)	-
NP76	ma	-	++
type IV collagen	pa	+/-	++
laminin	pa	++	+

ma = monoclonal; pa = polyclonal antibodies; ++ = strong positive; + = positive; +/- = weakly positive; - = negative; pc = pericellular

2.3.4 Digestion of frozen sections

Cryostat sections were pretreated with various enzymes or reagents that cause elution of masking proteins or swelling of the tissue.

No differences were observed using hyaluronidase, acetic acid or urea/GuHCl. After protease treatment with pepsin, trypsin or pronase the immunoreactivity of 1042/1043 was retained whereas the immunoreactivity with the other MA disappeared.

However, when bacterial collagenase (*Clostridium histolyticum*) was used, the immunoreactivity of 1042 and 1043 disappeared but the reactivity with the other MA remained, suggesting that these antibodies are directed against non-collagenous epitopes.

2.4 Discussion

BM all share the same basic structure, consisting of a lattice of type IV collagen and laminin (1). However, in view of the various functions of BM, cell or organ specific components may be expected to exist. The study of BM components, however, is difficult as they are linked in a complex and largely insoluble network with type IV collagen (2).

Furthermore, presumed and as yet unknown specific components might be present in small amounts only, which might remain undetected or be lost during purification and chemical analysis (21). An alternative approach to establish the presence of specific com-

ponents is the use of hybridoma technology. Immunization with intact BM might yield BM specific MA through screening of hybridoma supernatants by immunohistochemistry on the immunogen (11, 13). In the present study, which further extends our earlier observations (22), native BM from human amnion and glomeruli were used as immunogens, and the hybridoma supernatants were tested on cryostat sections of amnion and kidney. Three hybridomas with a BM restricted staining pattern: 1052, 1053 and 1065, obtained after immunization with amniotic BM were isolated. One hybridoma, which reacts with almost all BM, MA 1087, was obtained after immunization with glomerular BM. These MA were further analyzed by immunohistochemistry. The immunoreactivity patterns were compared with those of MA 1042 and 1043, which are directed against human type IV collagen (15). MA 1052 and 1053 showed no immunoreactivity on BM of mesodermal tissues, e.g. around blood vessels, muscle cells, fat cells or in the kidney, whereas almost all ectodermal and endodermal epithelial BM were stained with these MA. However, in the gastrointestinal tract heterogeneous epithelial BM staining was observed. Only BM in the superficial segments of the crypts and the top of the villi stained described previously (22). The heterogeneous staining pattern remained after digestion, elution or swelling of the tissue, suggesting that the heterogeneity is not due to masking of the epitope (4, 21, 24). Furthermore, the epitopes of MA 1052 and 1053 were only detected in the BM adherent to the dermis after chemical splitting of the skin. These findings suggest that MA 1052 and 1053 detect a BM component with a basic structural role or a function in anchoring the basement membrane to the interstitial tissue, comparable to type VII collagen, the collagenous component of the anchoring fibers (9). Alternatively, components which exclusively localize in ecto- and endodermal epithelial BM, could be involved in mediating the barrier function of these epithelia. Isolation of these components and detailed chemical analysis might further elucidate their nature and function in the BM.

MA 1087 shows a pattern of immunoreactivity resembling that of MA 1042 and 1043, which are directed against type IV collagen. On chemically split skin MA 1042, 1043 and 1087 localize mainly in the BM segment adherent to the dermis, suggesting localization in the lamina densa. Our immunohistochemical results on chemically split skin are in agreement with immuno-electron microscopic observations, which localize laminin predominantly in the lamina lucida and type IV collagen predominantly in the lamina densa (25-27). These results suggest that MA 1087 could be directed against type IV collagen. Contrary to the epitopes of MA 1042 and 1043, however, the 1087 epitope is insensitive for bacterial collagenase, which might indicate that this MA reacts with the non-collagenous globular part of type IV collagen, so called the NC1 domain (28-31).

MA 1065 reacts mainly with ectodermally derived epithelial BM and does not react with endodermally derived epithelial BM. The only exception to this is the BM of the oesophageal and tracheal epithelial lining. Furthermore pericellular staining occurs around basal cells of the epidermis and cornea. In chemically split skin MA 1065 shows immunoreactivity only in the epidermal segment. The pericellular localization suggests that the antigen detected by MA 1065 might be involved in intercellular adhesion and/or adhesion of the basal cells to the basement membrane.

In conclusion, the newly produced set of MA to BM confirm that BM in various anatomic sites have unique characteristics. The use of intact BM as immunogen in combination with the hybridoma technology for the production of MA and immunohistochemistry for MA selection is a useful approach to study new BM components. This approach may be used to further unravel the BM structure.

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Chapter 3

Biochemical and immunohistochemical characterization of monoclonal antibodies against the NC1 globular domain of type IV collagen*

*Cleutjens JPM, Beek C, van Breda Vriesman PJC, Bosman FT.
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3.1 Introduction

The basement membrane (BM) is a specific compartment of the extracellular matrix, which is in close juxtaposition with epithelial and endothelial cells and tightly surrounds some mesenchymal cells e.g. fat, muscle and Schwann cells (1).

The major structural component of the BM is type IV collagen (2, 3) of which the triple helix is composed of at least two different α chains ($\alpha 1(IV)$ and $\alpha 2(IV)$). The amino-terminus of the triple-helical monomer contains a disulfide-rich domain which combines with three other amino-terminal ends to form a tetramer (4-6). The carboxy-terminus of the molecule contains a large globular domain, which binds to the globular domain of another type IV collagen molecule, partly by disulfide bonds and partly by covalent cross-linking, to form the NC1 (non-collagenous) domain (7). The NC1 domain can be released from the triple helix by digestion with bacterial collagenase. When isolated from this digest the NC1 domain appears to be a hexameric structure with a molecular weight of approximately 170 kD, which can be separated into monomers and dimers by incubation with 6 M guanidinium hydrochloride, urea, dilute acetic acid or sodium dodecyl sulphate (8).

The NC1 domain has received considerable attention in connection with the Goodpasture syndrome (9). Goodpasture syndrome is clinically characterized by pulmonary hemorrhages followed by renal insufficiency, and immunohistochemically characterized by diffuse linear staining of pulmonary and glomerular BM. This linear staining is caused by binding of IgG autoantibodies to the BM. Although the epitopes of the Goodpasture autoantibodies can be found in all BM, mainly the kidney and the lung are affected. Increased reactivity with Goodpasture autoantibodies has been observed with denatured BM antigens, which suggests that normally cryptic antigenic determinants in human kidney and lung can be exposed and induce autoantibody formation, resulting in the Goodpasture syndrome. Recent studies have shown that the Goodpasture antigen is sequestered inside the hexameric structure of the NC1 domain of type IV collagen (8). The antigen can be detected by immunoblotting, using autoantibodies from Goodpasture patients, which react with monomeric and dimeric bands of hexamers released from type IV collagen by collagenase digestion.

We have recently raised BM reactive monoclonal antibodies (MA) through immunization with amniotic and glomerular BM (10). By immunohistochemistry some MA (1087) showed uniform linear staining of all BM, whereas others (1052 and 1053) showed heterogeneous BM staining. The latter MA reacted only with BM in ecto- and endodermal organs, and in the gastro-intestinal tract only with superficial BM of crypts and villi. The epitopes of these MA appeared to be insensitive to bacterial collagenase. This finding led us to compare the immunoreactivity of MA 1052, 1053 and 1087 with sera from patients with Goodpasture syndrome and anti-glomerular BM (anti-GBM) glomerulonephritis. Patients with anti-GBM nephritis resemble those with Goodpasture syndrome in having IgG autoantibodies bound to the glomerular BM. In contrast to the Goodpasture syndrome,

the pulmonary hemorrhages in anti-GBM glomerulonephritis are caused by volume overload secondary to renal insufficiency. In the present report we describe the biochemical and immunohistochemical properties of these MA.

3.2 Materials and methods

3.2.1 Isolation of the basement membranes

Human amniotic membranes, obtained directly after delivery, were separated into chorion and amnion. The amniotic membrane was washed in phosphate buffered saline pH 7.4 and mechanically treated with a rubber policeman to remove blood and adhering chorionic membranes. For immunization the membranes were denuded by chemical and mechanical treatment as described by Liotta et al. (11). In short, amnion was washed one hour in distilled water with 2 mM N-ethyl maleimide (NEM), extracted one hour with 1 M NaCl, 20 mM EDTA, 2 mM NEM and finally one hour with 4% de-oxycholate. After each step the membranes were mechanically treated with a rubber policeman to remove amniotic epithelial cells and most of the interstitial stroma.

Human glomerular BM extracts were kindly provided by Dr. B. van den Heuvel (Nijmegen). These were prepared according to the procedure described by Langeveld et al. (12). In short human kidney cortex was sequentially sieved on several mesh sizes. Glomeruli were obtained at mesh sizes between 90 and 125 μm . The glomeruli were lysed in 0.1% NaN_3 and 1 mM EDTA, centrifuged and subsequently incubated 5 h with 4% sodium de-oxycholate, treated 2.5 h with deoxyribonuclease (59 Kunitz units/ml) washed with distilled water and lyophilized.

3.2.2 Monoclonal antibodies

Monoclonal antibodies were obtained after immunization with native amniotic or glomerular BM, as extensively described in chapter 2 (10). Antibody production by hybridomas was detected immunohistochemically on frozen sections of the immunogens. Productive clones (1052, 1053 and 1087) were recloned until monoclonal by limiting dilution. MA to human type IV collagen (1042 and 1043), isolated from human placenta by pepsin digestion (13) were obtained and described extensively in chapter 9.4.

3.2.3 Immunohistochemistry

Frozen sections of human tissues were air-dried and fixed in acetone. The sections were washed with PBS and incubated with hybridoma culture supernatant or using sera of patients with autoantibodies to glomerular BM. After washing the sections were incubated with peroxidase labeled conjugate and peroxidase activity was visualized by incubation with diaminobenzidine as described in chapter 9.2.

3.2.4 Pretreatment of tissue sections

To unmask hidden epitopes, frozen sections (4 μ m) of human kidney cortex were pre-treated during 5 min at different temperatures (RT, 50 °C or 100 °C) with 6 M urea, 6M guanidiniumhydrochloride or acetic acid pH 3.0 (8). Frozen sections of human amnion and human kidney cortex were digested with 0.5 mg/ml bacterial collagenase in 50 mM Tris, 0.36 mM CaCl₂ (pH 7.6) at 37 °C for 30 min, 1 hr, 2 hr, 4 hr and overnight and subsequently stained as described under immunohistochemistry (chapter 9.2), the digest however was spotted onto nitrocellulose, dried and treated 3 times 10 min with PBS, 0.1% bovine serum albumin and subsequently stained with the monoclonal antibodies as described under immunohistochemistry (chapter 9.2).

3.2.5 Patients and sera

The patients derived from the Regional Working Party on Renal Diseases and included one patient with Goodpasture syndrome and 5 patients with rapidly progressive glomerulonephritis caused by anti-GBM antibodies. The diagnosis anti-GBM glomerulonephritis was based on:

- a. linear deposition of IgG along the GBM with diffuse extra capillary crescent formation.
- b. the presence of anti-GBM antibodies in the serum by radio-immuno-assay (RIA) and by enzyme-linked-immunosorbent-assay (ELISA) (25-27).
- c. the absence of pulmonary hemorrhages prior to irreversible renal insufficiency.

The patient with the Goodpasture syndrome developed pulmonary hemorrhages followed by renal insufficiency due to diffuse crescentic glomerulonephritis after one month and associated with linear deposition of IgG autoantibodies alongside the GBM. This patient showed no anti-GBM antibodies by RIA and ELISA in the serum. Patient data, data on renal immunopathology and patient serums were kindly provided by Prof. van Breda Vriesman, Dept. of Immunology, University of Maastricht.

3.2.6 Collagenase digestion

Collagenase digestion was performed as described by Kleppel et al. (14). Human amniotic membranes or human kidney cortex were washed with phosphate buffered saline (PBS) pH 7.4, 1 mM phenylmethylsulfonylfluoride (PMSF), 2 mM n-ethyl maleimide (NEM), and subsequently the tissue was minced using a Ultraturrax-grinder. The tissue fragments were centrifuged 10,000 g at 4 °C and extracted during 24 h, 4 °C with PBS, 0.5 M NaCl, PMSF, NEM pH 7.4, centrifuged and treated 48 hr with 3% acetic acid, PMSF, NEM, 10 μ g/ml pepstatin A and centrifuged. The pellet was freeze dried and digested 24 h, 37 °C with 10 U/mg collagenase (type VII collagenase, *Clostridium histolyticum*; Sigma) diluted in 50 mM Tris (pH 7.6), 0.2 M NaCl, 2 mM CaCl₂. The digest was centrifuged at 16,000 g for 30 min and the supernatant was dialyzed against water and subsequently freeze-dried.

3.2.7 Electrophoresis

Isolated proteins were run on 10% SDS polyacrylamide gels (15), and subsequently electrobotted to nitrocellulose paper (16) as described in chapter 9.5. Reductive cleavage was performed by incubating 360 μ l of a solution of amnion collagenase digest (10 mg/ml) in 0.1 M Tris, 5 mM EDTA pH, 8.2 with 40 μ l 0.1 M dithiothreitol, dissolved in the same buffer. After 1, 3 and 6 h incubation at 37 °C, 20 μ l was taken out of the solution and the reduction was stopped by adding 20 μ l 50 mM N-ethylmaleimide in 0.1 M Tris pH 7.4 (24). Prior to electrophoresis SDS was added to an endconcentration of 1 % (w/v) and the reaction mixture was heated for 5 min at 95 °C.

For isoelectric focusing, samples were run on a 5% polyacrylamide gel (3% cross-linking), containing 10% glycerol and 6% ampholytes (0.33% (v/v) ampholytes pH 4-6, 0.33% (v/v) ampholytes pH 5-7, 0.66% (v/v) ampholytes pH 9-11, 4.66% (v/v) ampholytes pH 3.5-10). The collagenase digest was run 3.5 h at 4 °C with 12 W constant power, and one lane with marker proteins (Serva, Heidelberg) as a reference. To check the pH gradient, 0.5 cm pieces were cut out the gel, incubated overnight with distilled water and subsequently measured. The focused proteins were blotted semidry to nitrocellulose paper.

3.3 Results

3.3.1 Immunohistochemistry

The MA obtained by immunization with amniotic membranes (1052 and 1053) showed a heterogeneous staining pattern, reacting exclusively with BM of epidermis and epidermal adnexa, such as sebaceous and sweat glands and hair follicles. Furthermore BM of glandular epithelium of breast and prostate, squamous epithelium of the tongue, oropharynx, oesophagus, and vagina, and ciliated epithelia of the trachea and bronchus as well as alveolar BM in the lung were stained. In the lung no reactivity occurred with the endothelial basement membrane. In the gastrointestinal tract epithelial BM of the superficial crypt (colon) or villous (small intestine) structures was observed, whereas deeper in the mucosa BM reactivity was absent. No reactivity was obtained with renal or vascular BM or with BM of fat-, muscle-, or Schwann cells.

Immunoreactivity could not be induced by tissue pretreatment with denaturing agents, such as guanidiniumhydrochloride, urea, SDS or acetic acid, nor by digestion with bacterial collagenase, whereas the dot spotted collagenase digest stained with MA 1052, 1053 and 1087.

MA 1087, obtained by immunization with human glomerular BM, showed staining of all BM except the central epithelial BM of the cornea. Staining of cryostat sections from a renal biopsy of a patient with Alport's syndrome with anti type IV collagen MA (1042 and

1043) and MA 1087, showed distinctly less intense immunoreactivity with 1087 than with 1042 and 1043. The staining intensity was especially less in the glomerular and tubular BM, whereas homogeneous staining of the endothelial BM was obtained with all MA.

On tissue sections of human amnion the immunoreactivity of MA 1052, 1053 and 1087 was retained after treatment with bacterial collagenase for 30 min until 4 hr. However, similar pretreatment of sections from kidney cortex showed that MA 1087 still reacted with the BM of this tissue, whereas with MA 1052 and 1053 even after severe digestion no immunoreactivity could be observed. After overnight collagenase digestion no immunoreactivity could be observed with any of the tissues and antibodies used due to disintegration of the tissue. However, when the digest of the sections was spotted onto nitrocellulose filters clear immunoreactivity could be observed with all MA on both tissues. The staining intensity diminished after overnight incubation, perhaps due to severe cleavage of the isolated hexamer by bacterial collagenase or by the effect of other enzymatic impurities in the digestion mixture. The epitopes of all three MA lost immunoreactivity by treatment with proteases such as pepsin, trypsin and pronase.

3.3.2 Immunochemistry

In an attempt to isolate the epitopes of the MA, amniotic membranes and kidney cortex were digested with collagenase, which releases the NC1 domains. The reactivity of the MA was tested after electrophoresis and immunoblotting of the digested protein. MA 1087, 1052 and 1053 showed identical patterns of reactivity after electrophoresis under unreduced conditions with amnion collagenase digest (Fig 1). Three major immunoreactive bands were found with a molecular weight of about 48, 42 and 23 kD respectively. In addition the collagenase digest of amnion showed two minor bands with a molecular weight of 95 and 86 kD. The digest of kidney cortex showed another low MW band of about 26 kD (Fig 2). The immunoreactivity with the 48 and 42 kD bands was observed with all three monoclonal antibodies and with both tissues. Under reducing conditions none of the above mentioned bands could be detected but one new band of 32 kD occurred, which stained with all three MA (Fig 3). A shift to higher molecular weight bands was observed after incomplete reduction. The collagenase digest from the amniotic BM was electrophoresed, blotted and stained with sera from patients with anti-GBM glomerulonephritis and Goodpasture syndrome. The staining pattern varied somewhat dependent on the antibody titer of the patient sera, but the sera of patients with anti-GBM glomerulonephritis (lane 1, 3-6) showed reaction with the 48, 42 and 23 kD bands, dependant on the dilution of the serum (Fig 4). The patient with Goodpasture syndrome did not show immunoreactivity with these bands. Two additional major bands were detected, with a molecular weight of about 36 and 20 kD, and also two minor bands with molecular weights between 80 and 98 kD.

After isoelectric focusing, the focused proteins were subsequently blotted onto nitrocellulose filters. All MA stained two bands intensely, with isoelectric points at pH 8.4 and 7.8, and furthermore a weaker staining band was observed with an isoelectric point of pH 7.6 (Fig 5).

Figure 1. Immunoblot after 10% SDS polyacrylamide gelelectrophoresis of unreduced collagenase digested human amniotic membranes.

Lane 1: immunoreactivity obtained with MA 1087,
 Lane 2: immunoreactivity obtained with MA 1052,
 Lane 3: immunoreactivity obtained with MA 1053,
 Lane 4: negative control.

D = dimeric bands with a MW of 48 and 42 kD,
 M = monomeric band with a MW of 23 kD.

Figure 2. Immunoblot after 10% SDS-polyacrylamide gelelectrophoresis of unreduced collagenase digest of: human kidney cortex (lane 1 and 3), collagenase digest of human amniotic membranes (lane 2 and 4).

The blots were respectively stained with:
 MA 1087 (lane 1 and 2),
 MA 1052 (lane 3 and 4).

Figure 3. Immunoblot after 10% SDS-polyacrylamide gelelectrophoresis of unreduced and reduced collagenase digest of human amniotic membranes and stained with MA 1087.

Lane 1: unreduced,
Lane 2-4: reduction with 0.1 M dithiothreitol for:
1 hour (lane 2),
3 hours (lane 3),
6 hours (lane 4).

Figure 4. Immunoblot after 10% SDS-polyacrylamide gelelectrophoresis of unreduced collagenase digest from amniotic membranes stained with:

patient sera with anti-GBM glomerulonephritis (lane 1 and 3-6),
patient serum with Goodpasture syndrome (lane 2),
MA 1087 (lane 7).

Figure 5. Immunoblot after isoelectric focusing in a 5% gel of collagenase digested amniotic membranes. Lane 1 = stained with MA 1087, Lane 2 = stained with MA 1052.

3.4 Discussion

At the carboxyterminal NC1-domains of two type IV collagen molecules these molecules are bound together (7). NC1 domains can be isolated from BM by bacterial collagenase digestion (14). Under these conditions the NC1-domain appears as a hexameric structure of which the monomers are bound together partly by disulfide bonds and partly by covalent cross-linking (7). The hexamer dissociates into dimers and monomers under acidic or denaturing conditions.

The NC1-domain has recently gained considerable attention through its association with the so called Goodpasture antigen (28). The Goodpasture syndrome is characterized by pulmonary hemorrhages and circulating autoantibodies, reactive with pulmonary alveolar and glomerular BM. With Goodpasture patient sera only the glomerular BM stains, unless tissues are pretreated with denaturing or acidic agents. The Goodpasture epitope is thought to reside inside the hexameric structure of the globular domain of type IV collagen, and denaturation of this structure is needed to expose the epitope (8, 17). Recently the reactivity of Goodpasture sera with a novel $\alpha 3$ chain of type IV collagen (18, 19) was reported. We have isolated three MA against human BM: MA 1087 reacting with almost all BM and MA 1052 and 1053 reacting uniformly with BM of ectodermal, and heterogeneously with BM of endodermal and not at all with BM of mesodermal origin (10). The epitopes of MA 1052, 1053 and 1087 are all susceptible to protease treatment but resistant to bacterial collagenase.

Treatment of tissue sections with denaturing or acidic agents or collagenase digestion did not induce immunoreactivity with MA 1052 and 1053 in mesodermal BM, whereas immunoreactivity with all three MA could be detected after dot spotting of this tissue section digest. This finding suggests that in order to expose the epitopes of MA 1052 and 1053 in mesodermal BM, these have to be released from the triple helical part of type IV collagen. Staining of a kidney section from a patient with Alports familial nephritis showed less immunoreactivity of MA 1087 in glomerular and tubular BM than with MA against the triple helical part of type IV collagen (1042 and 1043) as was described for Goodpasture patient sera by Kashtan et al. (20). These findings suggest that at least the epitope of MA 1087 could be associated with the Goodpasture antigen.

In order to resolve this matter we performed a series of immunoblotting experiments with our MA as well as with sera from patients with autoimmune glomerulonephritis. After SDS-gelelectrophoresis all MA showed reactivity with bands with molecular weights of 48, 42 and 23 kD, which resemble the D2* dimers and M2* monomers of the NC1 domain of type IV collagen (21-23, 29). Staining of higher molecular weight bands (80-98 kD) can be explained as undissociated tetramers of the NC1 domain. Using sera with autoantibodies to glomerular BM we also observed these dimers (48 and 42 kD and sometimes also the monomer (23 kD). Some patient sera stained additional bands (36 and 20 kD), which suggests that also other BM components can become target in this autoimmune disease (8).

Although on immunoblots MA 1052, 1053 and 1087 showed the same immunoreactivity pattern, different patterns were noted in immunohistochemistry experiments. MA 1052 and 1053 only stained a limited range of epithelial BM whereas 1087 stained all BM, except the central epithelial corneal BM. Even collagenase treatment did not induce immunoreactivity with MA 1052 and 1053 in the kidney, whereas the dot spotted digest or electrophoresed collagenase digest showed reactivity with MA 1052, 1053 and 1087. This discrepancy points towards an interesting phenomenon. Although the NC1-domain epitopes occur in BM of kidney and amnion, in the kidney the supramolecular structure of the BM in situ masks the epitopes of MA 1052 and 1053. This finding suggests that in different BM variations in assembly of the same basic BM components exists. This could be due to heterogeneous combinations of the 3 to 4 different α -chains of type IV collagen (19). Another explanation could be that both epitopes are present, but in some organs the epitopes of MA 1052 and 1053 are masked by other BM components attached to type IV collagen. In conclusion, our three monoclonal anti BM-antibodies identify epitopes in the NC1-domain which reveal characteristic heterogeneity in the supramolecular assembly of type IV collagen in the BM.

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Chapter 4

Absence of type IV collagen in corneal epithelial basement membrane*

*Cleutjens JPM, Havenith MG, Vallinga M, Bosman FT.
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4.1 Introduction

Basement membranes (BM) are ubiquitous extracellular matrix structures which connect epithelial cells with the adjacent connective tissue. In addition, individual adipose-, muscle-, and Schwann cells are enveloped by a BM (1). BM consist of various components, of which type IV collagen (2), laminin (3), heparan sulphate proteoglycan (4), nidogen/entactin (5, 6) are localized up to now in all BM. Epidermal BM furthermore contains components which are not localized in all BM, such as bullous pemphigoid-, cicatricial pemphigoid-, and epidermolysis bullosa acquisita antigen (7), type VII collagen (8, 9) and hitherto unidentified components recognized by monoclonal antibodies e.g. KF-1 (10), AF1 and 2 (11) and LDA-1 (12).

The cornea is a specialized ectodermal structure. Newsome et al. (13), Guillaumin et al. (14), and Nakayasu et al. (15) reported that type IV collagen and laminin occur throughout the entire human corneal epithelial BM. However, in the rabbit (16, 17), guinea pig and chicken (18, 19) and in man (20, 21, 22) it has been shown that in the central corneal epithelial BM type IV collagen is largely, if not completely, absent albeit it is abundantly present in conjunctival epithelial and vascular BM. Also in the stroma isolated structures are reactive with antibodies directed against type IV collagen and laminin. These structures may be remnants of embryonic vessels (16) or Schwann cell BM (17).

In the present study we have investigated whether BM heterogeneity occurs in the human cornea, similarly to the staining pattern for type IV collagen in rabbit and chicken cornea. Therefore, the epithelial BM composition was studied by immunohistochemistry. Corneas were stained with a panel of polyclonal- as well as monoclonal antibodies directed against type IV collagen, type VII collagen, laminin and heparan sulphate proteoglycan. Furthermore the ultrastructure of the epithelial-stromal interface was studied in central and peripheral parts of the cornea.

4.2 Materials and methods

4.2.1 Antibodies

Antibodies used in this study were monoclonal (1042, 1043 and 1087) and polyclonal anti-type IV collagen antisera (23 and chapter 2), monoclonal (4E10) and polyclonal anti-laminin (57 E/6) antibodies (24), a monoclonal anti-type VII collagen antibody (9) and a monoclonal anti-heparan sulphate proteoglycan antibody. The specificities of these antibodies are outlined in chapter 9.4.

4.2.2 Tissues

Eight adult human corneas were obtained at autopsy. The corneas were partly fixed in 4% buffered formalin and embedded in Paraplast or mounted in OCT-compound (Lab-Tek products, Naperville, USA) and snap frozen in isopentane quenched in liquid nitrogen.

4.2.3 Immunohistochemistry

Frozen corneas were cryostat sectioned and fixed in acetone, treated with H_2O_2 in methanol to block endogenous peroxidase activity and incubated with monoclonal- or polyclonal antibodies. After washing with PBS the sections were incubated with peroxidase labeled conjugate. Immunoreactivity was visualized by incubation with diaminobenzidine- H_2O_2 substrate. Paraffin sections were deparaffinized, rehydrated and blocked for endogenous peroxidase. To obtain immunoreactivity with type IV collagen and laminin these sections required treatment with 0.1% pepsin (Boehringer, Mannheim, FRG) in 0.1 N HCl (30 min, RT). Further immunohistochemical procedures were identical to those described for frozen sections (chapter 9.2 and 9.3).

4.2.4 Tissue pretreatment

Frozen as well as paraffin sections were pretreated with various enzymes in order to unmask the immunoreactivity of BM components. For paraffin sections we used 0.1% pepsin (Boehringer, Mannheim, FRG) in 0.1 N HCl (30 min, RT) or 0.04% pronase (Boehringer, Mannheim, FRG) in 50 mM Tris-HCl pH 8.0 (30 min, 37 °C) or 0.1% hyaluronidase (Cooper Biomedical) in 0.1 M sodiumphosphate buffer (0.15 M NaCl, pH 4.5, 30 min, RT). Frozen sections were pretreated with various dilutions of the same enzymes. Other sections were pretreated with various concentrations of guanidiniumhydrochloride 4 or 6 M in 50 mM sodium acetate, 50 mM EDTA, pH 6.5, 30 min, RT, or with 0.1 or 0.5 M acetic acid, 30 min, RT (25, 26). Frozen sections were preincubated with reducing (1 mM sodium periodate) and oxidizing (0.5% sodium borohydrate) agents diluted in PBS with different time intervals (27, 28).

4.2.5 Electron microscopy

Corneal specimens of 1 mm³ from the central and peripheral part were fixed in 2.5 % glutaraldehyde in 0.1 M sodium phosphatebuffer pH 7.2, postfixed 1 hr in 1 % OsO_4 , dehydrated in a graded series of alcohols and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate.

4.3 Results

Cryostat and paraffin sections (the latter after pretreatment with pepsin) were stained with antibodies against BM constituents (Table 1, Fig 1).

Type IV collagen was abundantly present in the epithelial BM of the limbus and conjunctiva and in vascular BM. The central corneal epithelial BM, however, lacked immunoreactivity with monoclonal- but also with polyclonal antibodies directed against human type IV collagen. As soon as Bowman's membrane appeared in the limbal/corneal transition, type

IV collagen immunoreactivity diminished and within a short distance disappeared completely (Fig 1B). Antibodies directed against other epidermal BM components such as laminin and heparan sulphate proteoglycan showed continuous reactivity of the conjunctival- and limbal epithelial/stromal interface and the corneal epithelial/Bowman's membrane interface, whereas Bowman's membrane itself showed no immunoreactivity with these antibodies. With polyclonal anti-laminin antisera, in addition to corneal epithelial BM reactivity, strong supranuclear intracytoplasmic staining was observed only in basal corneal cells. To determine whether the central corneal epithelial BM lacked type IV collagen, or alternatively the epitopes were masked by other constituents such as proteoglycans or glycoproteins, paraffin- and cryostat sections were pretreated with various concentrations of enzymes e.g. pepsin, trypsin, collagenase/dispase and hyaluronidase (17), with various concentrations of guanidinium hydrochloride to extract proteoglycans or with acetic acid which causes swelling or conformational changes within type IV collagen containing fibrils. Other tissue sections were pretreated with reducing or oxidizing agents. Despite these attempts to unmask the type IV collagen epitopes, no immunoreactivity with the monoclonal and polyclonal antibodies to type IV collagen could be obtained in the central epithelial corneal BM.

Table 1. Immunoreactivity for BM components in the human cornea

antibody	corneal ep BM	limbal/con- junctival BM	ep	Bowman's membrane	Descemet's membrane		
					stromal face	central face	endothelial face
ma type IV collagen	—	+	—	—	+	—	—
pa type IV collagen	—	+	—	—	+	+	—
ma laminin	+	+	—	—	—	—	+
pa laminin	+	+	*	—	—	—	+
ma type VII collagen	+	+	—	—	—	—	—
ma HSPG	+	+	—	—	—	—	+

ma = monoclonal; pa = polyclonal antibody; HSPG = heparan sulphate proteoglycan; * = supranuclear staining in basal cells; ep = epithelium

Figure 1. Immunoperoxidase staining pattern of frozen human corneal epithelial BM. Conjunctiva/limbus (A, D, G), Central cornea (B, E, H), Descemet's membrane (C, F, I), were stained using antibodies to type IV collagen (A, B, C), laminin (D, E, F) and type VII collagen (G, H, I). Scale bar = 20 μ m.

Figure 2. A. Electronmicrograph of peripheral (conjunctival) human corneal epithelial BM. B. Electronmicrograph of central human corneal BM. E = epithelium; l.l. = lamina lucida; l.d. = lamina densa; BO = Bowman's membrane. Scale bar = 0.5 μ m.

Descemet's membrane reacted with antibodies to laminin and heparan sulphate proteoglycan, exhibiting a linear staining pattern only with the endothelial face of this membrane. In contrast, with monoclonal anti-type IV collagen antibodies only the stromal face of Descemet's membrane reacted. With polyclonal anti-type IV collagen antisera the entire Descemet's membrane showed weak immunoreactivity in addition to the stromal staining pattern. The immunohistochemical results were identical on frozen and cryostat sections. Ultrastructurally, differences were observed between the peripheral and central corneal BM. Normal lamina lucida and lamina densa were observed in limbal and conjunctival BM (Fig 2A). In the central area of the cornea the BM showed a lamina lucida, however the lamina densa was absent or less distinct in comparison to the peripheral part of the cornea. Electron dense depositions directly underlying the lamina lucida were considered to represent parts of hemidesmosomes. Elsewhere the lamina lucida was in direct contact to the Bowman's membrane (Fig 2B).

4.4 Discussion

Type IV collagen is regarded as the structural backbone of all BM (1, 29). Present data however show that some parts of the BM may not contain type IV collagen and/or be reactive with some antibodies to this type of collagen. Within a single continuous (corneal) BM heterogeneous reactivity occurs with antibodies to human type IV collagen. In the central corneal epithelial BM, type IV collagen could not be detected with the used antibodies, whereas this component was abundantly present in the limbal-, conjunctival-, and vascular BM. This is in contrast to earlier findings in which the human cornea showed a linear staining for type IV collagen of the whole corneal epithelial BM (13-15). In the human cornea, we found that conjunctival and limbal BM have a normal ultrastructural appearance with a lamina lucida and lamina densa, whereas in the adult central corneal BM the lamina densa is not as distinct as in the periphery of the cornea or indeed totally absent. The lamina lucida appeared to be directly attached to the Bowman's membrane. These findings support the contention that type IV collagen is predominantly localized in the lamina densa (30, 31). Immuno-electron microscopic investigation with antibodies to type IV collagen, which localize type IV collagen throughout lamina densa and lamina lucida, as well as model studies on BM reconstitution from isolated BM components (type IV collagen, laminin and heparan sulphate proteoglycan) suggest that these components are codistributed, although they do not rule out the possibility that the relative proportions of these components may vary (32).

For the absence of type IV collagen immunoreactivity in the central cornea several explanations should be considered. Firstly, type IV collagen could be completely absent. In this situation the BM scaffold could consist of a component, different from type IV collagen, to which the other components are attached. Secondly, an altered type IV collagen might be deposited, which does not react with our anti-type IV collagen antibodies, as was earlier suggested by Fitch et al. (19). Thirdly, a BM might be altogether absent. In this situation,

Bowman's membrane could take over its function. Other BM components, such as laminin and heparan sulphate proteoglycan were detected in the interface between epithelium and Bowman's membrane. It is therefore unlikely that the entire BM is missing; more likely the function of type IV collagen is taken over by another component. Fourthly, it is possible that in the central corneal region type IV collagen determinants are masked by other BM components such as glycoproteins or proteoglycans (16). However, pretreatment of the tissue with various enzymes or agents which cause elution of proteoglycans, swelling of the tissue or conformational changes within the BM components did not restore immunoreactivity with any of the antibodies used in this study. Fifthly, the absence of humoral and stromal stimuli for the production of BM components might explain the lack of type IV collagen. This might be envisioned as basal corneal epithelial cells are implanted on an acellular and avascular specialized stroma (Bowman's membrane) and are consequently devoid of a normal epithelial-stromal transition. Finally, central basal corneal cells are highly differentiated cells, which might have lost the capability of type IV collagen production. Central corneal epithelial cells are not capable of replication in culture, whereas peripheral/limbal corneal epithelium maintains the potential of duplication in vitro (33, 34). Schermer et al. (34), and Rodrigues et al. (35) postulated the existence of corneal stem cells in the limbus. This contention is supported by the pattern of expression of a 64 kD keratin, which is correlated with specific epithelial differentiation and shows a suprabasal localization in the limbus, and a basal localization in the central cornea (Cleutjens et al., unpublished observations). Furthermore the observed centripetal migration of corneal epithelial cells from the limbal area and the higher mitotic activity of the limbal cells compared to the central corneal cells supports this hypothesis.

Antibodies to laminin and heparan sulphate proteoglycan reacted with all BM and only differences in staining intensity have been observed. In our experiments the central corneal BM showed less intense staining for laminin and heparan sulphate proteoglycan compared to the limbal/conjunctival BM staining, which is similar to the findings for type IV collagen in the human cornea by others (13, 14). The presence of type VII collagen and the absence of type IV collagen immunoreactivity in the central corneal region implies that type IV collagen is not the only possible junctional element in linking the carboxyl-terminal domain of type VII collagen in anchoring plaques and BM (36). In the cornea, type VII collagen must bind to other BM components. The attachment site could be the hemidesmosome because hemidesmosomes and anchoring fibril collagen appear synchronously during development and wound healing (37).

Heterogeneity in BM composition was found also in Descemet's membrane. With antibodies to laminin and heparan sulphate proteoglycan only the endothelial face of Descemet's membrane showed delicate linear staining. In contrast, monoclonal antibodies to type IV collagen showed only immunoreactivity with the stromal face, whereas polyclonal antisera stained the stromal face with occasional faint staining of the central part of Descemet's membrane. These findings make it likely that Descemet's membrane is a true BM formed by endothelial cells (34). This membrane may in addition contain other compo-

nents which are not generally found in BM (39). In contrast to Descemet's membrane, Bowman's membrane is a modified stromal membrane and it did not react with various antibodies to BM components.

From these results we conclude that central human corneal epithelial BM lacks type IV collagen. Consequently, the basic structure of this BM is different from that of other BM.

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Chapter 5

Umbilical cord stromal cells are myofibroblasts: isolation, immunohistochemical and ultrastructural characterization*

*Cleutjens JPM, Vallinga M, Beek C, ten Kate J, Bosman FT.
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5.1 Introduction

The myofibroblast is a much disputed member of the mesenchymal cell family. Cells with structural and functional characteristics of fibroblasts and smooth muscle cells were first described in experimentally induced wounds and granulation tissue (1). The myofibroblast is morphologically characterized by a fibroblastic-type rough endoplasmic reticulum and peripheral parallel cytoplasmic filaments with dense body condensations characteristic of myofilaments. Basement membrane-like structures are found around the myofibroblast as a cell-coat or as discontinuous plaques. In close apposition to this basement membrane-like material often hemidesmosome-like attachment bodies are observed. Myofibroblasts display extensive cell-matrix interactions: intracellular actin-filaments are attached to a hemidesmosomal plaque, whereas collagen fibers are arranged extracellularly in anchoring strands (2) and extracellular fibronectin into a fibronexus (3). Furthermore myofibroblasts are attached to each other by desmosome-like structures. When the cell contracts, the whole tissue architecture will be altered. Myofibroblasts contract and relaxate on the same pharmacologic stimuli as smooth muscle cells. Along with fibroblasts, these cells produce collagen and elastin (4, 5) and conceivably various other extracellular matrix components.

Myofibroblasts play an important role in some pathologic conditions connected with e.g. tissue repair, such as wound healing or the desmoplastic reaction in neoplasia. In neoplasia, when the desmoplastic response is inhibited by dehydropoline, invasive growth and metastasis are increased and this is accompanied by a decrease in collagen synthesis (6). In coculture experiments of myofibroblasts with tumor cells, the growth of the tumor cells was inhibited due to a still unidentified synthesized pronase susceptible protein (7).

The origin of myofibroblasts is still under discussion. They might be derived from a mesenchymal stem cell or differentiate from fibroblasts and/or smooth muscle cells (4, 5). Studies of the biology of myofibroblasts would be greatly facilitated by availability of model systems in which these cells could be manipulated. We therefore investigated the possibility to culture myofibroblasts from umbilical cord stroma. The present report describes these cells in the umbilical cord stroma, their tissue culture and their immunophenotype and ultrastructure.

5.2 Materials and methods

5.2.1 Antibodies

Monoclonal and polyclonal antibodies against type IV collagen, laminin, desmin, vimentin, fibronectin, factor VIII, actin and myosin were used in this study. The characteristics and source of the antibodies are extensively specified in chapter 9.4.

5.2.2 Tissue culture

Human umbilical cords were obtained directly after delivery. The stromal part of this tissue was excised and washed several times in phosphate buffered saline (PBS) with 50 Units/ml penicillin and 50 μ g/ml streptomycin (pen/strep). The little fragments of stromal tissue were cultured in Dulbecco's modified Eagles minimum essential medium supplemented with 10 % fetal calf serum and pen/strep using a 37 °C incubator with 5 % CO₂. After one to two weeks cells grew out from these explants. These cells were removed by trypsinization and cultured under pen/strep free culture conditions. To culture human skin fibroblasts, human dermis was used as explant. Cells were cultured either on tissue culture plastics, on 1 % gelatin coated glass slides or in and on type I collagen lattices. Type I collagen lattices were prepared as described previously (8, 9, chapter 9.8) by mixing a cold stock solution of type I collagen isolated from human placenta (4 mg/ml in 0.1 % HAc) with culture medium and 0.1 N NaOH to neutralize the pH. The endconcentration of type I collagen was 0.7, 1 or 2 mg/ml. Reaggregation took place as soon as the solution was neutralized and brought to 37 °C.

5.2.3 Electron microscopy

Tissue fragments of approximately 1-2 mm³ were fixed in 2.5 % glutaraldehyde in 0.1 M sodium phosphatebuffer pH 7.2, postfixed 1 hr in 1 % OsO₄, dehydrated in a graded series of alcohols and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate.

5.2.4 Immunohistochemistry

Western blots, dot blots, paraffin- and frozen sections were stained with an indirect immunoperoxidase method as described in chapter 9.2. Briefly, frozen sections were air-dried and fixed in acetone. Paraffin sections were first deparaffinized. The sections were washed with PBS and incubated with the specific antibodies. After washing with PBS the sections were incubated with peroxidase labeled conjugate. After washing with PBS, peroxidase activity was visualized by incubation with diaminobenzidine-H₂O₂ substrate.

Before applying the specific antibody to western and dot-blot the nitrocellulose paper was treated with PBS and 1 % Bovine serum albumin or 0.3 % Tween 20 to prevent aspecific binding of the antibodies.

5.2.5 Electrophoresis and western blotting

Culture supernatants were precipitated with 4.5 M NaCl or 50 % ammoniumsulphate, dialyzed against 0.1 M Tris-HCl and electroblotted (10) after 5 % polyacrylamide gel electrophoresis (11, chapter 9.5).

After western blotting the strips of nitrocellulose paper were stained with polyclonal anti-type IV collagen and laminin antisera as described under immunohistochemistry (chapter 9.2).

5.2.6 Immunoaffinity purification

Monoclonal anti-human type IV collagen antibody (1043) was coupled to Affigel-prot A (Bio-rad). Culture supernatant was used undiluted or precipitated with 50 % ammonium sulphate pH 7.4, dialyzed against phosphate buffered saline and incubated overnight at 4 °C on a roller bank. After washing 4 times (with 50 mM Tris, 150 mM NaCl, pH 7.4), the affinity purified material was eluted with 0.02 M HAc, 150 mM NaCl, dialyzed against distilled water and freeze dried.

5.2.7 Northern blotting

Cultured cells were isolated by trypsin digestion and cytoplasmic RNA was isolated by resuspending the cell pellet (12). The nuclear pellet was removed, the supernatant extracted with phenol-chloroform and the RNA was precipitated in ethanol. Total RNA was separated by electrophoresis in agarose-formaldehyde gels and blotted overnight onto a gene-screen filter. The blot was air dried, baked, and prehybridized during in a solution containing NaCl, Na-citrate, Na-phosphate, polyvinyl pyrrolidone, bovine serum albumin, ficoll, denaturated salmon sperm DNA and formamide. Hybridization of RNA-cDNA was carried out in the same solution containing the labeled type IV collagen cDNA probe (13). The type IV collagen probe was labeled with the random primer labeling method (14, 15) with ³²P-dCTP. After hybridization the filter was washed, dried and autoradiographed using Kodak X-Omat films. This method is extensively outlined in chapter 9.6.

5.2.8 In situ hybridization

4 µm frozen sections were mounted on RNase free coated slides, fixed with freshly prepared 4% paraformaldehyde and washed with PBS, baked, washed again and treated with Triton X-100. After washing in PBS the sections were incubated with proteinase K in a buffer containing Tris, and EDTA. The sections were fixed again with 4% buffered paraformaldehyde, washed with PBS and treated with acetic anhydride in triethanolamine and furthermore incubated with formamide in 2x SSC. ³⁵S-dCTP labeled type IV collagen probe (13) was prepared according to the random primed labeling method using a Boehringer labeling kit (14, 15). 15 µl probe/hybridization mixture containing 300,000 cpm was applied to each section. The sections were subsequently covered with 22x22 mm siliconized coverslips and sealed with rubber cement. Hybridization mixture contained yeast t-RNA, salmon sperm DNA, deionized formamide, Tris-HCl, NaCl, EDTA, Denhardt's mixture, Dextran sulphate, and DTT. After 12-16 h incubation at 50 °C, the coverslips were removed and the slides were washed with SSC. Following these steps the slides were dehy-

drated in graded series of ethanol, air dried and coated with Kodak NTB-2 nuclear track emulsion and exposed for varying periods of time. The exposed slides were developed in 1:1 Kodak D-19 developer fixed, and counterstained with hematoxylin, dehydrated and mounted. Parallel sections were hybridized with a ^{35}S labeled empty plasmid as a negative control. This method is extensively described in chapter 9.7.

5.3 Results

5.3.1 Cell morphology

The fibroblast-like cells in the stroma of the umbilical cord are characterized electron microscopically by a fibroblast-type of rough endoplasmic reticulum, an undulating nucleus, dense bodies, myofilaments, focal pericellular basement membrane depositions, and desmosome-like cell contacts (Fig 1 and 2).

Following *in vitro* explantation of umbilical stroma, spindle shaped cells grew out within two weeks. The stromal cells *in vitro* were flattened, elongated and sometimes sprouting, with a clear nucleus containing one or two nucleoli. The cells could be kept in continuous culture and could be subcultured in standard medium under standard conditions. A problem was cryopreservation of the cells in order to store them for longer periods.

Figure 1. Electronmicrograph of myofibroblasts in the umbilical cord stroma. Scale bar = 0.1 μm .

Figure 2. Electronmicrograph of myofibroblasts in the umbilical cord stroma. A: Peripheral myofilaments (MF) and discontinuous depositions of basement membrane-like material around the cell membrane (BM). B: Desmosome-like contacts between two myofibroblastic cells (*). Scale bar = 0.1 μ m.

Figure 3. Immunoperoxidase staining pattern of a frozen section of human umbilical cord stained with MA 1042, directed to human type IV collagen. A: Overview, with (1) smooth muscle cell BM, (2) stromal cell BM and (3) amniotic BM. B: Higher magnification of the stromal cells stained with anti type IV collagen monoclonal antibodies. Scale bar = 10 μ m.

Figure 4. Immunoblot after 5% polyacrylamide gelelectrophoresis of affinity purified type IV collagen, isolated from tissue culture supernatant of human umbilical cord stromal cells in vitro, and stained with lane 1: polyclonal anti-type IV collagen antiserum lane 2: polyclonal anti-laminin antiserum.

Figure 5. Northern blot of mRNA isolated from umbilical cord stromal cells *in vitro*, hybridized with a type IV collagen cDNA probe (lane 1) and as a control mRNA from human skin fibroblasts hybridized with the same probe (lane 2).

Several cryoprotectants, including 10 % glycerol, DMSO and 100% foetal calf serum were tested but after thawing, reculturing of the cells appeared to be impossible. When cultured in a lattice of type I collagen, the cells ultrastructurally resembled the stromal umbilical cord cells *in vivo*. They showed an undulating nucleus, rough endoplasmic reticulum, myofilaments, dense bodies, and focal extracellular basement membrane depositions. The cells contracted the lattice within a few days, similar to human skin fibroblasts.

5.3.2 Immunophenotype

Staining of the umbilical cord frozen sections with a panel of antibodies showed that the stromal cells stained with vimentin, desmin, α and γ actin and smooth muscle myosin, suggesting that they are mesenchymal cells with contractile properties. Furthermore intracytoplasmic and/or extracellular BM components, such as type IV collagen and laminin could be observed (Fig 3), whereas this appeared to be the only cell type in the umbilical cord which produces fibronectin. When cultured on gelatin coated glass slides the umbilical cord stromal cells showed by immunohistochemistry with this panel of antibodies (Table 1) the same pattern of immunoreactivity as found for the stromal cells in the umbilical cord.

Table 1. Immunoreactivity of umbilical cord cell types *in vivo* and *in vitro*

Antibodies	<i>in vivo</i>						<i>in vitro</i>			
	Endo		SMC		MF		Amnion		UC	
	BM	cell	BM	cell	BM*	cell	BM	cell	cell	
ma 1042/1043	+	—	+	—	++	+	+	—	+(pc)	
ma 1087	+	—	+	—	+	+	+	—	+(pc)	
pa type IV collagen	+	—	+	—	++	+	+	—	+(pc)	
ma 4E10	+	—	+	—	+	+	+	—	+(pc)	
pa laminin	+	—	+	—	+	+	+	—	+(pc)	
pa type I collagen	—	—	—	—	—	+	—	—	+	
ma vimentin	—	+	—	+	—	+	—	+	++	
ma desmin	—	—	—	+	—	+	—	—	+/-	
pa desmin	—	—	—	+	—	+	—	—	+	
ma α actin	—	—	—	++	—	++	—	—	++	
ma α + γ actin	—	—	—	++	—	++	—	—	++	
pa sm myosin	—	—	—	+	—	+	—	—	+	
ma pan myosin	—	—	—	++	—	+	—	—	++	
ma myosin R11D10	—	—	—	—	—	—	—	—	—	
ma factor VIII	—	+	—	—	—	—	—	—	—	
ma fibronectin	—	—	—	—	—	+	—	—	++	

ma = monoclonal; pa = polyclonal; endo = endothelium; SMC = smooth muscle cell; MF = myofibroblast; UC = umbilical cord stroma cell; BM = basement membrane; * = basement membrane-like material; sm = smooth muscle; pc = pericellular immunoreactivity

5.3.3 Production of BM components

Tissue culture supernatant from cultured stromal cells was affinity purified using a monoclonal antibody to type IV collagen (clone 1043). Using this method laminin copurified with type IV collagen as demonstrated by electrophoresis followed by western blotting, using polyclonal antisera to type IV collagen and laminin (Fig 4). In the cultured cells type IV collagen mRNA was detected by northern blotting. After hybridization with the type IV collagen cDNA probe, two bands were observed just above the 28S ribosomal RNA band, whereas no hybridization was obtained with mRNA from normal skin fibroblasts (Fig 5). *In situ* hybridization on tissue sections of umbilical cord and cytospin preparations of cul-

Figure 6. In situ hybridization of cultured human umbilical cord myofibroblasts on gelatin coated glass slides and hybridized with a type IV collagen cDNA probe. Scale bar = 10 μ m.

tured cells showed strong labeling of the cytoplasm of the cultured cells (Fig 6), whereas in tissue sections in addition to the stromal cells some amniotic epithelial cells, smooth muscle cells and endothelial cells were labelled.

5.4 Discussion

Myofibroblasts are mesenchymal cells which share some characteristics of fibroblasts and smooth muscle cells. These cells show a well developed rough endoplasmic reticulum, nuclear indentations, myofilaments, pinocytotic vesicles, attachment bodies and focal deposits of basement membrane components (4, 5). Myofibroblasts have an important function in wound healing (1). In granulation tissue and desmoplastic tissue reactions, this cell type is responsible for wound contraction and for the production of new "embryonal" type III collagen which is later replaced by type I collagen (16-18). The origin of the myofibroblast is still unclear. More specifically, the question arises whether these cells develop from a mesenchymal stem cell or alternatively are fibroblasts or smooth muscle cells in a specific functionally activated condition.

In order to shed more light on this question we studied the stromal cells of the umbilical cord. This stroma is in direct continuity with that of the placental villi, which contains numerous myofibroblasts (19). From these findings it can be assumed that the stromal cells in the umbilical cord are myofibroblasts. We found by electron microscopy cells with an

undulating nucleus with one or two nucleoli, fairly abundant focally dilated rough endoplasmic reticulum, peripheral bundles of microfilaments with dense bodies, attachment bodies and focal extracellular deposition of BM-like material.

In the cells the expression of BM components could be observed by immunohistochemistry and hybridocytochemical detection of mRNA for type IV collagen. Immunohistochemically, we could detect intra- and extracellular laminin and type IV collagen in tissue sections of umbilical cord as well as *in vitro* in monolayer or in collagen lattice cultures. Type IV collagen and laminin could furthermore be isolated from the tissue culture supernatant and *in vitro* abundant type IV collagen mRNA could be detected by *in situ* hybridization. This contrasted with skin fibroblasts, which did not produce any BM component. Pihlajaniemi et al. (13) used "normal" primary human fibroblasts as type IV collagen producing cells. This discrepancy might be explained by assuming that these cells, isolated as normal fibroblasts, can be activated *in vitro* in such a way that they are capable to produce type IV collagen. Since we used the same probe as Pihlajaniemi et al. (13) it is tempting to speculate on a relationship between normal fibroblasts, identified by production of type I collagen and activated fibroblasts or myofibroblasts producing type IV collagen. Which factor causes this phenotypic shift has still to be investigated. These results prove that the stromal cells are not fibroblasts, although they share with fibroblasts the production of fibronectin and interstitial collagen types (I and III) as can be observed from the immunohistochemical results and as described by others (16-18).

The production of BM components could suggest that the stromal cells are smooth muscle cells. Smooth muscle cells usually express desmin, smooth muscle myosin and α actin (20-22) and these cytoskeletal proteins also occurred in the stromal cells. Two features, however, argue against the smooth muscle nature of the stromal cells. Firstly, they express fibronectin, which is not found in smooth muscle cells. Secondly, the BM was patchy and interrupted and the cells also produced interstitial collagens. Based upon these findings, we feel justified to conclude that the stromal cells of the umbilical cord are myofibroblasts. Our results demonstrate that these cells may express more phenotypic characteristics of smooth muscle cells (20, 21) than reported previously (23) (e.g. expression of smooth muscle myosin and α actin). What the exact developmental relationship is between the different mesenchymal cells remains to be established.

Their role in the production of extracellular matrix, the regulation of contractile properties and their function in pathological processes, especially tissue repair and desmoplastic reaction in neoplasia, might be elucidated further in *in vivo* experiments.

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Chapter 6

Origin of basement membrane type IV collagen in xenografted human epithelial tumor cell lines*

*Cleutjens JPM, Havenith MG, Vallinga M, Beek C, ten Kate J, Bosman FT.
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6.1 Introduction

Basement membranes (BM), at the interface between epithelia and the underlying stroma, are composed of macromolecules such as type IV collagen (1), laminin (2, 3) and heparan sulphate proteoglycan (4). Using specific antibodies to one or more of these constituents it has been shown that in many carcinomas a discontinuous BM is deposited at the epithelial-stromal interface. It has been suggested that the ability of cancer cells to form/degrade a BM may be associated with invasive and metastatic behaviour (5-10) and may be correlated with patient survival (11). It is generally assumed that epithelial BM are at least partly a product of the epithelial cells (12, 13). Several investigators have demonstrated that *in vitro* epithelial tumor cells may continue to produce BM components (14-22). However evidence has been presented that mesenchymal cells are able to produce BM components and may therefore contribute to the formation of the BM. Kühl et al. (23) and Sanderson et al. (24) showed by cocultivation of mouse muscle fibroblasts and quail skeletal myoblasts, that the myoblast BM is of mouse origin. Similarly, combination of chick mesenchyme and rat endoderm resulted in formation of a BM of mesenchymal origin (25). However, experimental data concerning BM origin in neoplasms *in vivo* is very scanty. Recently, Damjanov et al. (26) studied the extracellular matrix of human tumor xenografts in nude mice using monoclonal anti-laminin antibodies, exclusively reactive with human laminin, and polyclonal anti-laminin antisera reacting with human as well as with murine laminin. In many of their xenografts, tumoral BM laminin appeared to be of human as well as murine origin. They concluded that laminin in the extracellular matrix of xenografted tumors is at least partly of stromal origin. In this report we xenografted four human tumor cell lines into nude rats. Two of the cell lines did not produce (human) type IV collagen *in vitro*, whereas the other cell lines were able to produce type IV collagen. After xenografting, the production of type IV collagen was immunohistochemically investigated with antibodies to human and host specific epitopes of type IV collagen, in order to gain insight in the possible contribution of epithelial and mesenchymal components in the assembly of type IV collagen molecules into a BM. *In situ* hybridization using a type IV collagen cDNA probe, recognizing human and mouse/rat type IV collagen mRNA, was used to investigate whether the observed immunohistochemical pattern correlates with the expression of mRNA coding for this protein.

6.2 Materials and methods

6.2.1 Tissues

Human cell lines derived from colonic adenocarcinoma (5583-E, HT-29), from transformed amniotic epithelium (WISH), and from an oral epidermoid carcinoma (KB) were used in this study. The characteristics of the 5583-E and HT-29 cell lines are described extensively elsewhere (27, 28). The WISH and KB cell lines were obtained from the Ameri-

can Type Culture Collection (ATCC). The characteristics of these cell lines were reported by Hayflick and Eagle (29, 30). Cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM, Flow Laboratories, Amstelsdam, Zwanenburg, The Netherlands) supplemented with 10% fetal calf serum (FCS, Boehringer, Mannheim, FRG), before xenografting.

Human type I collagen was isolated from placenta as described elsewhere (31). Cells were cultured in or on human type I collagen lattices (1 or 2 mg collagen/ml) as described by Nusgens (32). Lattices were sometimes populated with human skin fibroblasts, which contract the lattice within a few days. For histological evaluation the collagen lattices were processed as described for the tissue samples.

6.2.2 Xenografting

All tumor cell lines were xenografted in nude mice (NMRI nu/nu supplied by Zentral Institut für Versuchstiere, Hannover, FRG) and nude rats (CD 1 nu/nu supplied by Charles River, Sulzfeld, FRG) by injecting tumor cell suspensions (1.5×10^6 cells in 100-200 μ l PBS) subcutaneously. After a period of 2-6 weeks solid tumors of 0.5-1 cm in diameter were obtained.

Xenografts were removed and samples of the xenografts and the collagen lattice cultures were snap frozen in isopentane quenched in liquid nitrogen and stored at -70 °C and/or fixed in 4% buffered formaldehyde and further embedded in paraffin.

6.2.3 Antibodies

Polyclonal cross-species reactive antisera were generated by immunization of rabbits with human type IV collagen isolated by pepsin digestion from human placenta as extensively described elsewhere (31). Monoclonal antibodies (1042 and 1043) were obtained after immunization with pepsin cleaved type IV collagen from human placenta (31). Monoclonal antibody 1043 is human specific, whereas 1042 reacts with human and rabbit type IV collagen (31). A polyclonal rabbit anti-mouse type IV collagen was kindly provided by Dr. M. Demarchez and Dr. D.J. Hartmann (33). This antibody was adsorbed several times with human type IV collagen, and was used in a 1:1000-1:2000 dilution.

Mouse monoclonal anti-human type VII collagen (NP76) was kindly donated by Dr. R. Burgeson. This antibody is human specific and reacts only with the epithelial BM of epidermis, trachea and oesophagus (34). Mouse monoclonal anti-human laminin (4E10) (35), is human specific and reacts with all BM. Rabbit polyclonal anti-mouse laminin obtained after immunizing rabbits with mouse EHS laminin (E/Y Laboratories, Sanbio, Uden, The Netherlands), is reactive with all BM. This polyclonal antiserum is cross-species specific and does not react with type IV collagen or fibronectin on dot-blots and western-blots. The characteristics and source of the antibodies are extensively specified in chapter 9.4.

6.2.4 Immunoblotting of culture supernatants

Supernatants of 5583-E, HT-29, WISH and KB cells were precipitated with 4M NaCl, dissolved in and dialyzed against SDS-sample buffer and run on a 5% SDS-polyacrylamide gel (36), followed by blotting onto nitrocellulose membrane (37) as described in chapter 9.5. The nitrocellulose filters were washed (3 times 10 min) with PBS and 0.05% Tween 20, and subsequently stained as described under immunohistochemistry (chapter 9.2).

6.2.5 Immunohistochemistry

In order to expose the antigenic sites, for all antigens paraffin tissue sections were pre-treated with pronase (Boehringer, Mannheim, FRG) when monoclonal antibodies were applied, or with pepsin (Boehringer, Mannheim, FRG) for the polyclonal antisera. On frozen sections the antibodies could be employed without enzymatic pretreatment. Sections were incubated for one hour with the primary antibodies and subsequently with peroxidase labeled rabbit anti-mouse IgG or peroxidase labeled goat anti-rabbit IgG (Dakopatts, Glostrup, Denmark) for the monoclonal and polyclonal antibodies respectively. After each incubation, sections were washed with PBS (3 x 5 min). Immunoreactive sites were visualized with diaminobenzidine. Finally, the sections were lightly counterstained with hematoxylin as described in chapter 9.2 and 9.3.

6.2.6 In situ hybridization

4 μ m frozen sections were mounted on RNase free coated slides, fixed with freshly prepared 4% paraformaldehyde and washed with PBS, baked, washed again and treated with Triton X-100. After washing in PBS the sections were incubated with proteinase K in a buffer containing Tris and EDTA. The sections were fixed again with 4% buffered paraformaldehyde, washed with PBS and treated with acetic anhydride in triethanolamine and furthermore incubated with formamide in 2x SSC. 35 S-dCTP labeled type IV collagen probe (38) was prepared according to the random primed labeling method using a Boehringer labeling kit (43, 44). 15 μ l probe/hybridization mixture containing 300,000 cpm was applied to each section. The sections were subsequently covered with 22x22 mm siliconized coverslips and sealed with rubber cement. Hybridization mixture contained yeast t-RNA, salmon sperm DNA, deionized formamide, Tris-HCl, NaCl, EDTA, Denhardt's mixture, Dextran sulphate, and DTT. After 12-16 h incubation at 50°C, the coverslips were removed and the sections were washed with SSC. Following these steps the sections were dehydrated in graded series of ethanol, air dried and coated with Kodak NTB-2 nuclear track emulsion and exposed for various periods of time. The exposed slides were developed in 1:1 Kodak D-19 developer, fixed, and counterstained with hematoxylin, dehydrated and mounted. Parallel sections were hybridized with an 35 S labeled empty plasmid as a negative control. This method is extensively described in chapter 9.7.

6.3 Results

6.3.1 Antibody specificity

By ELISA the polyclonal anti-human type IV collagen antiserum did not react with types I and III collagen and showed only a slight reaction with type V collagen (Fig 1A). After solid phase immunoadsorption with type IV collagen, the antiserum only detected type IV collagen. Reactivity with type I, III and V collagen could not be detected with the monoclonal antibodies (Fig 1B + C). In immunoelectroblotting experiments the polyclonal antiserum showed immunoreactivity with several reduced and non-reduced type IV collagen bands, in the same pattern as the Aurodyne stain of the reduced and non-reduced type IV collagen (Fig 2 lanes 2-5). However, the monoclonal antibodies both showed immunoreactivity with a single band in the high molecular region of non-reduced type IV collagen and very weak immunoreactivity with a single band in the low molecular weight region (Fig 2 lanes 6-9).

Immunohistochemical experiments further substantiated the specificity of these antibodies. On a wide variety of human tissues both polyclonal and monoclonal anti-type IV collagen antibodies reacted exclusively with BM in an identical pattern. On sections of normal tissues from various other species (including Balb/c and NMRI nu/nu mice or rats) polyclonal anti-type IV collagen antisera showed a similar pattern of reactivity.

Figure 1. ELISA testing of reactivity of anti-type IV collagen antibodies with:

- human type I collagen
- human type III collagen
- ▲ human type IV collagen
- ▽ human type V collagen

A. rabbit polyclonal anti-type IV antiserum

B. 1042, mouse monoclonal anti-human type IV collagen antibody

C. 1043, mouse monoclonal anti-human type IV collagen antibody

Figure 2. Western blot of SDS-PAGE separated collagens. Lane 1: reduced human type I collagen stained with Aurodye. Lane 2 and 3: reduced and non-reduced human type IV collagen stained with Aurodye. Lane 4 and 5: reduced and non-reduced human type IV collagen reacted with polyclonal anti-human type IV collagen. Lane 6 and 7: reduced and non-reduced human type IV collagen reacted with monoclonal 1042. Lane 8 and 9: reduced and non-reduced human type IV collagen reacted with monoclonal 1043

The two monoclonal anti-type IV collagen antibodies, however, did not show any reactivity with tissues from these species. The polyclonal antiserum to mouse type IV collagen, absorbed with human type IV collagen, was not reactive with BM on frozen sections of human tissues, but showed intense immunoreactivity with BM in mouse tissues (Fig 3). The polyclonal anti-mouse type IV collagen antiserum was also reactive with BM in rat tissues in a pattern identical to that of the cross-species reactive polyclonal anti-human type IV collagen antiserum, as described above.

6.3.2 Type IV collagen detection in cancer cell lines

Immunohistochemical studies of 5583-E or HT-29 cells grown in artificial collagen lattices did not reveal reactivity with any of the polyclonal or monoclonal anti-type IV collagen antibodies.

Figure 3. Immunoreactivity on frozen sections from human skin (A, C, E) and rat skin (B, D, F) with: polyclonal anti-human type IV collagen antiserum (A, B), polyclonal anti-mouse type IV collagen antiserum (C, D), monoclonal (1042) anti-human type IV collagen antibody (E, F). Scale bar = 10 μ m.

Figure 4. Frozen sections of rat xenografts of tumor cell-line 5583-E (A,B,C) and KB (D,E,F) (immunoperoxidase).

A and D: cross species reactive polyclonal antiserum to type IV collagen.

B and E: mouse and rat specific polyclonal antiserum to type IV collagen.

C and F: human specific monoclonal antibody to type IV collagen (MA 1042).

Scale bar = 10 μ m.

On immunoblots of precipitated tissue culture supernatants of 5583-E and HT-29 immunoreactivity with antibodies to type IV collagen did not occur. In contrast, pro $\alpha 1$ - and pro $\alpha 2$ -chains of type IV collagen were detected in supernatants of WISH and KB cell lines. WISH and KB cells, cultured in collagen lattices, showed extensive granular intracytoplasmic and/or pericellular immunoreactivity for type IV collagen with polyclonal as well as monoclonal anti-type IV collagen antibodies. However, BM like structures could not be identified (chapter 7).

6.3.3 Type IV collagen detection in xenografts

Rat xenografts were used for immunohistochemistry in order to avoid background staining when using mouse monoclonal antibodies on mouse tissues in an indirect peroxidase technique.

In xenografts of 5583-E and HT-29 cells immunohistochemical studies with cross-species reactive polyclonal anti-human type IV collagen antiserum showed reactivity with epithelial as well as vascular BM (Table 1). The epithelial BM were irregular and discontinuous, with many tumor cell nests lacking a BM altogether. In 5583-E and HT-29 xenografts, however, both monoclonal antibodies did not show any reactivity for type IV collagen (Fig 4). Using the polyclonal anti-mouse type IV collagen, however, the staining pattern was identical with the cross-species reactive polyclonal antiserum.

Table 1. Immunoreactivity and in situ hybridization on frozen sections of xenotransplanted tumor cell lines

cell line	anti-type IV collagen			anti-laminin		<i>in situ</i> hybridization	
	pa cross	pa mouse/rat	ma hum	ma hum	pa cross	stroma	cells
5583-E	+	+	—	—	+	+	—
HT-29	+	+	—	—	+	+	—
KB	+	+	+	+	+	+	+
WISH	+	+	+	+	+	+	+

hum = human specific; cross = cross species reactive; mouse/rat = mouse/rat specific; cells = tumor cells; ma = monoclonal; pa = polyclonal

Using the monoclonal antibody to laminin no BM staining was observed, whereas BM-like structures could be visualized using the cross-species reactive polyclonal antiserum. No reactivity was observed using the monoclonal antibody to type VII collagen. In WISH and KB xenografts reactivity for type IV collagen with the monoclonal antibodies in a BM pattern could be demonstrated clearly (Fig 4).

Figure 5. In situ hybridization on frozen sections of rat xenografts of tumor cell line 5583 (A, B) and KB (C, D) with a type IV collagen cDNA probe.

A and C: hybridized with a type IV collagen cDNA probe.

B and D: negative control, hybridized with a labeled empty plasmid.

Scale bar = 10 μ m.

Not only human specific type IV collagen but also human laminin and type VII collagen could be demonstrated in these xenografts. BM of vascular structures in the xenografts did not react with the monoclonal anti-type IV collagen antibodies.

The polyclonal antiserum specific for mouse/rat type IV collagen and the cross-species reactive anti-human type IV collagen antiserum showed identical staining in all the xenografts. These antibodies also stained the BM of blood vessels, Schwann cells, muscle cells and fat cells.

6.3.4 In situ hybridization

The results of our *in situ* hybridization experiments are summarized in Table 1. Using a type IV collagen cDNA probe in *in situ* hybridization experiments on frozen sections of the xenotransplants of 5583-E and HT-29, we observed an intense hybridization reaction with the host stromal cells adjacent to the human tumor cells, but not with the tumor cells (Fig 5).

Experiments on frozen sections of KB and WISH xenotransplants showed a slight but distinct hybridization reaction with tumor cells and also with stromal cells (Fig 5). The used cDNA probe also recognized rat type IV collagen mRNA, because in all xenografts labeling was noticed of basal cells of the rat epidermis and also of some mesenchymal cells of the dermis adjacent to the epidermal BM or surrounding the xenografted tumor cells (Fig 6).

Figure 6. In situ hybridization on a frozen section of rat epidermis with a type IV collagen cDNA probe. Scale bar = 10 μ m.

6.4 Discussion

In xenografts of human tumor cell lines in nude mice or rats the interaction *in vivo* between neoplastic epithelial cells and mesenchymal stromal elements can be investigated. For example, the origin of the BM can be traced when antibodies which discriminate between human and murine BM components are employed in immunohistochemical studies. Using this approach Holmstrup (40) studied the immunophenotype of laminin and type IV collagen in BM of normal human oral mucosa, transplanted into nude mice. He found that species cross-reactive polyclonal antisera against laminin and type IV collagen reacted equally well with vascular and epithelial BM whereas human specific monoclonal antibodies reacted intensely with vascular BM but only weakly or not at all with epithelial BM. Holmstrup concluded that under these conditions oral mucosal cells participate in the deposition of BM at the epithelial/stromal interface and that the vascular supply of the transplant is of mixed human and murine origin.

Demarchez et al. (33) studied revascularization of human skin transplants in nude mice, applying murine and human specific anti-type IV collagen antibodies and anti-HLA-DR antibodies, to identify the origin of the BM and vascular endothelium respectively. They found a shift from human to murine endothelium and a subsequent change from human to mouse type IV collagen in the subendothelial BM. Kühl et al. (23) cocultured mouse myoblasts with chicken fibroblasts and found deposition of chicken type IV collagen in the BM around mouse myoblasts. Damjanov et al. (26) xenografted a lung adenocarcinoma and a yolk sac carcinoma, both producing laminin *in vitro*, and a hepatoblastoma and a hepatocellular carcinoma, both lacking this characteristic, into nude mice. Using species cross-reactive and human specific anti-laminin antibodies it was shown that the epithelial BM were of murine or mixed human/murine origin. These findings clearly illustrate that in the deposition of BM surrounding single cells or epithelial, endothelial, mesothelial sheets interstitial stromal cells may participate.

In this context we studied the origin of BM in human tumor cell lines, xenografted into nude mice and rats, using species cross-reactive as well as human specific and mouse or rat specific anti-type IV collagen antibodies.

To this end we used two cell lines which do and two cell lines which do not produce laminin and type IV collagen *in vitro*. The human colon cancer cell lines 5583-E (27) and HT-29 (28) in immunohistochemical experiments did not produce type IV collagen. By *in situ* hybridization no type IV collagen mRNA could be detected in these cells. Furthermore, in immunoblots of tissue culture supernatants type IV collagen was not detected. Nevertheless, in xenografts of these cells in nude mice and rats fragments of BM could be detected by immunohistochemistry, using a polyclonal species cross-reactive anti-type IV collagen antiserum. Using mouse and rat specific anti-type IV collagen antibodies, we could demonstrate the type IV collagen to contain mouse or rat specific but not human specific epitopes. Furthermore we could demonstrate type IV collagen mRNA in the stromal cells but not in the (xenografted) carcinoma cells. These findings prove that type IV collagen in the epithelial BM in xenografts of 5583-E and HT-29 cells is deposited by stromal cells.

Two other cell lines, KB (derived from a human squamous cell carcinoma) (30), and WISH (derived from *in vitro* transformed amnion epithelium) (29), *in vitro* produced type IV collagen (39). By immunohistochemistry intracytoplasmic type IV collagen immunoreactivity could be detected and by immunoblotting this BM component could be identified in the tissue culture supernatant. Furthermore by *in situ* hybridization we could detect type IV collagen mRNA in these cells. When xenografted into nude mice and rats these cell lines gave rise to tumors with extensive BM deposition. In these BM human as well as murine type IV collagen epitopes could be detected by immunohistochemistry. Furthermore, *in situ* hybridization using a type IV collagen cDNA probe (38) demonstrated type IV collagen mRNA in the epithelial and in the stromal cells. In these xenografts, therefore, the epithelial BM contained components derived from the epithelial cells as well as from stromal cells.

In situ hybridization experiments on rat skin using the type IV collagen cDNA probe showed that type IV collagen mRNA is present in the basal cells of the epidermis as well as in the underlying dermal cells. This observation suggests that normal epithelial BM may be also composed of epithelial and stromal components. Type IV collagen probes discriminating between human and rat type IV collagen (mRNA) might elucidate these findings observed in these xenografts, and additionally could give answer to the questions concerning turnover and expression rates of the different type IV collagen molecules.

Which stromal cells could be responsible for the production of the epithelial BM components? Additional experiments in neoplasms and in normal tissues are necessary to resolve this question. It is tempting to speculate, however, that myofibroblasts are involved. Myofibroblasts play an important role in tissue repair and the desmoplastic tissue response (41, 42) and are capable to produce a variety of BM components. Furthermore, in neoplasms often a desmoplastic reaction can be observed which consists largely of myofibroblasts which deposit large amounts of BM material in the extracellular matrix.

In conclusion, our results demonstrate that in immune-deficient mice or rats the BM surrounding the xenografted human cancer cells is (partly) derived from murine/rat and therefore stromal cells and partly tumor cell derived. It is suggested that in this situation but probably also in normal BM stromal myofibroblasts participate in the deposition of the BM at the epithelial/mesenchymal transition.

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Chapter 7

In vitro production and deposition of basement membrane components*

*Cleutjens JPM, Vallinga M, Beek C, Bosman FT.
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7.1 Introduction

The basement membrane (BM) is a specialized domain of the extracellular matrix, which connects epithelial cells with the underlying or surrounding interstitium (1). A type IV collagen lattice provides the basic structure of all BM. The only exception is the central corneal epithelium, where a type IV collagen lattice does not occur at the epithelial/stromal interface (2).

It is generally accepted that BM are deposited by the adjacent cells (3-5). In tissue culture, epithelial cells have been found to produce (6-9) and occasionally also deposit BM components into BM-like structures (10-13). Dissociated epidermal cells, however, usually do not deposit a BM *in vitro* (14). Some epithelial tumor cells do and others do not produce BM components *in vitro* (9). Recombined chick mesenchyme and rat endoderm, however, shows formation of a BM of chick i.e. mesenchymal origin (15). In xenotransplants of human carcinoma cells BM are deposited which contain elements of stromal (host)/as well as tumor cell (human) origin (16, 17). These findings indicate that epithelial BM may be of epithelial or stromal or combined origin and are deposited probably as a result of a specific interaction between epithelial and stromal cells. What exactly regulates the deposition of a BM is unknown. Involvement of extracellular matrix components is likely in view of the experiments which show that on a plastic substratum cultured mammary or epidermal cells do not deposit BM-like material, whereas on a type I collagen substratum or in a type I collagen gel patches of BM-like material are deposited pericellularly (13, 14, 18-20). In reviewing the literature a striking finding is that many cell types appear to produce BM components *in vitro*, but do not deposit a BM-like structure. Xenografting of such cells, however, often yields neoplastic carcinoma cell nests surrounded by a usually irregular and discontinuous but linear deposit of BM material.

In this study we have investigated the capacity of neoplastic epithelial cells to produce and deposit a BM *in vitro* under a variety of circumstances. Special attention was paid to the question whether or not interaction with fibroblasts, myofibroblasts or endothelial cells might induce the deposition of a BM.

7.2 Materials and methods

7.2.1 Cell lines

The cell lines used are listed in Table 1.

Table 1. Main characteristics of the used cells

cell type/type	source	reference
5583-E	colon carcinoma	Verstijnen et al. (21)
HT-29	colon carcinoma	Fogh et al. (22)
KB	epitheloid carcinoma	Eagle (23)
WISH	transformed amnion	Hayflick (24)
fibroblasts	human skin	
myofibroblasts	human umbilical cord	chapter 5
endothelial cells	human umbilical cord	

7.2.2 Tissue culture

Most cells were cultured in Dulbecco's modified Eagles minimum essential medium supplemented with 10 % fetal calf serum in a 37 °C incubator with 5 % CO₂.

Endothelial cells were cultured in RPMI/M199 medium with 20 % newborn calf serum, 20 U/ml Heparin (25) and 88 µg/ml endothelial cell growth factor (26). In an attempt to induce extracellular matrix (ECM) production the cells were cultured with ascorbic acid (50 µg/ml), endothelial cell conditioned culture supernatant (10 % of total volume), or epidermal growth factor (EGF, Gibco Laboratories, Chaygrin Falls, Ohio) (concentration 1, 5 and 10 ng/ml). Furthermore, the cells were cultured on tissue culture plastics, 1 % gelatin coated glass slides and in or on type I collagen lattices. Type I collagen lattices were prepared according to Bell et al. (27) and Nusgens et al. (28) by mixing cold stock solution (4 mg/ml type I collagen isolated from human placenta solubilized in 0.1 % HAc) with culture medium and 0.1 N NaOH to neutralize the pH. The final concentration of type I collagen was 0.7, 1 or 2 mg/ml. Reaggregation took place as soon as the solution was neutralized and brought to 37 °C (chapter 9.8). These lattices were populated with fibroblasts or myofibroblasts and/or endothelial cells in order to create a connective tissue like substrate *in vitro*.

7.2.3 Isolation of denuded amniotic membranes

Human amnion was separated from chorion and the amniotic BM was isolated by chemical and mechanical treatment as described by Liotta et al. (29). In short amnion was washed one hour in distilled water and 2 mM N-ethylmaleimide (NEM), extracted one hour with 1 M NaCl, 20 mM EDTA, 2 mM NEM and finally one hour with 4% de-oxycholate. After each step the membranes were mechanically treated with a rubber policeman to remove amniotic epithelial cells and most of the interstitial stroma. The membranes were suspended in a metal ring and sterilised by washing in PBS supplemented with penicillin/streptomycin, before culturing cells on the surface.

7.2.4 Xenografting

Tumor cells were subcutaneously transplanted into nude rats (Athymic nu/nu) and harvested when the tumor reached 0.5-1 cm in diameter. Tumor material was fixed in 4 % buffered formaldehyde and embedded in paraffin or frozen in OCT-compound (Lab-Tek products, Naperville, USA) with isopentane quenched in liquid nitrogen. Tissue sections of 4 μ m were cut and used for staining.

7.2.5 Dot- and immunoblotting

Tissue culture supernatants were precipitated with 4.5 M NaCl or 50 % ammonium-sulphate (pH 7.4), dialyzed against 0.1 M Tris-HCl (pH 8.0) and electroblotted (30) after 5 % polyacrylamide gelelectrophoresis (31, chapter 9.5). The supernatants were also spotted onto nitrocellulose sheets by applying 100 μ l culture supernatant on the paper using a Bio-dot apparatus (Bio-rad, Utrecht, The Netherlands). After western and dot blotting immunostaining was performed as described under immunohistochemistry.

7.2.6 Northern blotting

Cultured cells were isolated by trypsin digestion. Nuclei were isolated (32). The nuclear pellet was removed, the supernatant extracted with phenol-chloroform and the RNA was precipitated in ethanol. Total RNA was separated by electrophoresis in agarose-formaldehyde gels and blotted overnight onto a genescreen filter. The blot was air dried, baked, and prehybridized with a solution containing NaCl, Na-citrate, Na-phosphate, polyvinyl pyrrolidone, bovine serum albumin, ficoll, denaturated salmon sperm DNA and formamide. Hybridization of RNA-cDNA was carried out in the same solution containing the labeled type IV collagen cDNA probe (33). The type IV collagen probe was labeled with the random primer labeling method (34, 35) with 32 P-dCTP. After hybridization the filter was washed, dried and exposed to a Kodak X-Omat film. This method is extensively outlined in chapter 9.6.

7.2.7 In situ hybridization

4 μ m frozen sections were mounted on RNase free coated slides, fixed with freshly prepared 4% paraformaldehyde and washed with PBS, baked, washed again and treated with Triton X-100. After washing in PBS the sections were incubated with proteinase K in a buffer containing Tris and EDTA. The sections were fixed again with 4% buffered paraformaldehyde, washed with PBS and treated with acetic anhydride in triethanolamine and furthermore incubated with formamide in 2x SSC. 35 S-dCTP labeled type IV collagen probe (33) was prepared according to the random primed labeling method using a Boehringer labeling kit (34, 35). 15 μ l probe/hybridization mixture containing 300,000 cpm was applied to each section. The sections were subsequently covered with 22x22 mm sili-

conized coverslips and sealed with rubber cement. Hybridization mixture contained yeast t-RNA, salmon sperm DNA, deionized formamide, Tris-HCl, NaCl, EDTA, Denhardt's mixture, Dextran sulphate, and DTT. After 12-16 h incubation at 50 °C, the coverslips were removed and the slides were washed with SSC. Following these steps the slides were dehydrated in graded series of ethanol, air dried and coated with Kodak NTB-2 nuclear track emulsion and exposed for varying periods of time. The exposed slides were developed in 1:1 Kodak D-19 developer, fixed and counterstained with hematoxylin, dehydrated and mounted. Parallel sections were hybridized with an ^{35}S labeled empty plasmid as a negative control. This method is extensively described in chapter 9.7.

7.2.8 Immunohistochemistry

Western blots, dot blots, paraffin- and frozen sections were stained by an indirect immunoperoxidase method. Frozen sections (4 μm) were air-dried and fixed in acetone. Paraffin sections were first deparaffinized. The sections were washed with PBS and incubated with the primary antibodies. After washing with PBS the sections were incubated with peroxidase labeled conjugate. After washing in PBS, peroxidase activity was visualized by incubation with diaminobenzidine- H_2O_2 substrate (chapter 9.2 and 9.3). Before applying the primary antibody to western blots and dot blots the nitrocellulose paper was treated with PBS and 1 % bovine serum albumin or 0.05% Tween 20 to prevent aspecific binding of the antibodies.

Antibodies used were human specific monoclonal antibodies to type IV collagen (1043 and 1042, the latter is also reactive with rabbit BM) (36); cross species reactive rabbit anti-human type IV collagen polyclonal antibodies (36); mouse monoclonal anti-human laminin antibody (4E10) provided by U. Wewer (37); polyclonal rabbit anti-mouse (EHS) laminin antibody, cross reactive with human laminin epitopes, prepared by immunizing rabbits intracutaneously with 100 μg laminin (E/Y Laboratories) as described in chapter 9.4.

7.2.9 Electron microscopy

Tissue fragments of approximately 1-2 mm^3 were fixed in 2.5 % glutaraldehyde in 0.1 M sodium phosphatebuffer pH 7.2, postfixed 1 hr in 1 % OsO_4 , dehydrated in a graded series of alcohols and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate.

7.3 Results

The production of BM components by cultured cells and their tendency to deposit these into a BM under various conditions were studied in 3 different cell types: tumor cells which do and others which do not produce BM components *in vitro* and myofibroblasts which under certain conditions deposit a BM-like structure *in vitro*.

Under standard conditions in tissue culture 5583-E and HT-29 cells, derived from human colonic carcinoma, did not produce type IV collagen. Immunocytochemistry of the cultured cells and immunoblots of culture supernatants did not show any immunoreactivity for type IV collagen. By *in situ* hybridization type IV collagen mRNA could not be detected in these cells. Addition of ascorbic acid, endothelial cell conditioned medium or EGF did not induce the cells to produce BM components. Culturing of these cells on amniotic BM, type I collagen or in type I collagen lattices with and without the addition of skin fibroblasts, umbilical cord myofibroblasts or umbilical cord endothelial cells did not alter this situation.

Under standard conditions WISH and KB cells produced detectable amounts of type IV collagen in the culture supernatant, as demonstrated by dot-blotting and western blotting as shown in Table 2 and Fig 1. On western blots the pro $\alpha 1$ and pro $\alpha 2$ -chain of type IV collagen could be detected using a polyclonal anti-type IV collagen antibody. By immunohistochemistry using antibodies to basement membrane components peri- and sometimes intracellular immunoreactivity was noted when these cells were grown on gelatin coated glass slides or centrifuged on glass slides (Table 3, Fig 2).

Granular extracellular staining was observed with monoclonal antibodies to type IV collagen and with polyclonal antibodies to type IV collagen and laminin also intracytoplasmic staining was observed. By northern analysis of WISH and KB cells, traces of type IV collagen mRNA could be detected. By *in situ* hybridization weak cytoplasmic labeling was found using the type IV collagen cDNA probe.

Table 2. Production of type IV collagen *in vitro* determined by northern, western, and dot-blotting

cell line/type	northern blot	western blot	dot blot
5583-E	—	—	—
HT-29	nd	—	—
WISH	+ / —	+ (185,170 kD)	+
KB	+ / —	+ (185,170 kD)	+
Myofibroblasts	+ +	nd	+ +

+ / — = moderate reactivity; + = positive reactivity; + + = very strong reactivity; nd = not determined; kD = molecular weight in kiloDalton

Figure 1. Immunoblot after 5% SDS-polyacrylamide gelelectrophoresis of 4.5 M NaCl precipitate of WISH culture supernatant.

Lane 1: stained with polyclonal anti-laminin antiserum;

Lane 2: stained with polyclonal anti-type IV collagen antiserum.

Table 3. Immunoperoxidase staining of frozen sections and cells grown on glass slides

cell type	lattice				glass
	in/on	+ fibroblasts	+ endothelial cells	+ myofibroblasts	
5583-E	—	—	—	—	—
WISH	—	—	—	—	+ gran
KB	—	—	—	—	+ gran
Myofibroblasts	+ (pc)	nd	nd	nd	+ pc/gran
Endothelial cells	—	—	nd	—	+ pc/gran

pc = pericellular; gran = granular; nd = not determined.

Figure 2. Cytospin of KB cells grown *in vitro* and stained with monoclonal anti-type IV collagen antibodies. Scale bar = 1 μm .

Figure 3. Umbilical cord stromal cells (myofibroblasts) grown on gelatin coated glass slides stained with monoclonal anti-type IV collagen antibodies. Scale bar = 10 μm .

Cocultivation of these cells in type I collagen lattices with myofibroblasts and endothelial cells did not alter this situation. The production of small amounts of type IV collagen remained but no formation of an extracellular BM-like structure could be demonstrated. KB and WISH xenografted into nude rats, showed discontinuous but linear BM fragments between the tumor cells and at the transition of tumor cells and host stroma (Table 4). Under standard conditions *in vitro* the umbilical cord stromal myofibroblasts produced large quantities of type IV collagen. This was detectable by immunoblotting in the tissue culture supernatant as well as by immunocytochemistry.

Table 4. Immunoperoxidase staining with human specific anti-type IV collagen monoclonal antibodies on frozen sections of rat xenografts

	between tumorcells	transition rat stroma/tumorcells
5583-E	—	—
HT-29	—	—
WISH	+ BM/gran	+ BM/gran
KB	+ BM/gran	+ BM/gran

BM = basement membrane like structures,, gran = granular

Strikingly, when the cells grew in multilayered sheets, in addition to granular intracytoplasmic staining linear extracellular immunoreactivity for type IV collagen was observed (Fig 3). By northern analysis large quantities of type IV collagen mRNA could be demonstrated and this result was confined by *in situ* hybridization. By electron microscopy of myofibroblasts cultured in a type I collagen lattice focal BM structures, including a lamina lucida and lamina densa, were observed.

7.4 Discussion

BM components are released into the culture medium by some cell lines cultured *in vitro* (6, 7, 9). However, the assembly of these components into a BM-like structure is rarely observed (8). When cells are grown on normal tissue culture plastics, the formation of a BM-like structure is never seen. However, some investigators observed deposition of BM-like structures when epithelial cells were cultured in or on collagen gels (13, 18, 19). This finding suggests that mesenchymal stroma may be one of the factors involved in the regulation of the functional activity of epithelial cells (20).

The present study addressed two questions. Firstly, whether mesenchymal cells could induce production of BM components in epithelial cells which do not produce these substances under standard conditions. Secondly, whether mesenchymal cells could induce assembly *in vitro* of the produced BM components into BM-like structures.

In order to answer these questions we first established that the colonic tumor cell lines 5583-E and HT-29 do not produce type IV collagen *in vitro*, neither by immunohistochemistry on cells grown on glass slides nor by electrophoresis of the culture medium followed by immunoblotting nor by *in situ* hybridization using a type IV collagen cDNA probe

(33). In contrast, the other two cancer cell lines KB and WISH produced type IV collagen *in vitro*. Pro α_1 and pro α_2 chains of type IV collagen were detected after electrophoresis and immunoblotting of culture media. Immunohistochemistry of cultured cells showed predominantly extracellular but also sometimes intracellular punctated immunoreactivity, suggesting that the BM components are produced and secreted but not deposited into BM-like structures. When xenografted into nude rats/mice all cell lines (5583-E, HT-29, KB, WISH) gave rise to tumors with clearly demonstrable BM structures, derived from murine stromal cells (17). In KB and WISH xenografts the BM are partly deposited by the tumor cells and partly by the stromal cells. When combined, these findings suggest that epithelial/stromal cell interactions might induce epithelial cells to deposit a BM-like structure.

BM assembly as such is not impossible *in vitro*. This is demonstrated by the myofibroblasts from the umbilical cord stroma which showed, when the cells grew in multilayers, BM-like structures similar to those seen *in vivo*.

Our experiments clearly show that cocultivation of 5583-E and HT-29 cells with umbilical cord myofibroblasts, skin fibroblasts or umbilical cord endothelium cannot induce these cells to produce BM components. Even cells, such as KB and WISH, which spontaneously produce BM components, cannot be induced *in vitro* by mesenchymal cells to assemble these components into a BM-like structure.

Our results lead to the following conclusions:

1. Some human cancer cells (5583-E, HT-29) do not release, whereas others (WISH, KB) do release BM components *in vitro*. Only umbilical cord myofibroblasts deposit a recognizable BM *in vitro*.
2. Human cancer cells xenografted into nude rats, give rise to carcinomas, in which BM are deposited around tumor cell nests.
3. Cocultivation with fibroblasts, myofibroblasts or endothelial cells neither induce 5583-E and HT-29 cells to produce BM components nor induce WISH and KB cells to deposit a structurally recognizable BM *in vitro*.
4. Although evidently epithelial/stromal interactions do play a role in the deposition of a BM structure, the factors which regulate this process remain unknown.

7.5 References

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Chapter 8

General discussion

8.1 Introduction

In this chapter the results will be discussed in a somewhat broader context. This will be done by postulating the following three questions:

- a. Is it likely that basement membranes (BM) contain more components than those presently known and if so, how can they be identified?
- b. Do all BM have a uniform morphology and structure?
- c. How is the epithelial BM deposited?

8.2 New BM components?

Several BM components have been isolated and characterized. The best characterized BM components are laminin, type IV collagen, heparan sulphate proteoglycan and entactin. However, it has been postulated that more different BM components will eventually be identified in the near future (1, 2), due to new isolation techniques and increasing interest in extracellular matrix research.

In animal models, such as the mouse EHS-tumor and rat parietal yolk sac carcinoma, cross-linking of newly synthesized collagen can be prevented by treatment of the animals with β -amino-propionitrile fumarate (BAPN) (3). This procedure facilitates the isolation of the BM components. In man, however, the BAPN approach can not be used. Consequently, isolation of human BM components is hampered by the strong cross-links which occur among and between the different BM components. Enzymatic cleavage is essential for isolation of human BM components (1, 4). This method, however, provides only fragmented information, comparable to the pottery fragments archeologists have to rely on in reconstructing images of the past. For example, pepsin cleavage of human BM, yields only the triple helical part of the molecule (4), whereas the globular NC1-domain is completely degraded. In contrast, bacterial collagenase only yields the globular NC1-domain and destroys the triple helix of type IV collagen (5). During these procedures components which occur in very low concentrations can remain undetected (6).

An alternative approach is the use of the hybridoma technology to generate monoclonal antibodies after immunization with native BM fragments. With this method, antibodies can be generated which might recognize new BM components, but also variations in the molecular and supramolecular structure of well known components, as described in chapters 2 and 3. However, the advantage of this approach is also its major drawback. The epitopes of the generated monoclonal antibodies tend to be largely conformational, which implies that they can only be detected in their native structure and, for example, not after fixation of the tissue. This is a serious handicap for immuno-electron microscopical investigations or for retrospective studies on formaldehyde fixed and paraffin embedded archival human tissues. Furthermore, isolation procedures might destroy the native conformation of the epitopes, and interfere with their immunoreactivity. The practical and scientific value of such a monoclonal antibody would be somewhat limited, because the biochemical

and physiological properties of the epitope can not be elucidated. An alternative approach towards isolation of human BM components would be the xenotransplantation of human tumor cells, which produce considerable amounts of BM components, such as adenoid cystic carcinoma or endodermal sinus tumour, into lathyrotic athymic animals (3). In lathyrotic animals the lysine derived cross-links, which are normally formed in the BM (mainly between type IV collagen molecules) do not occur. This approach allows the isolation of BM components by mild extraction methods, which might lead to the isolation of new and/or intact human BM components.

8.3 Do all BM have a uniform structure and morphology?

Most BM share the same ground structure and type IV collagen forms the structural backbone to which other components like laminin and heparan sulphate proteoglycan can attach (7). However, in normal BM differences in thickness can be observed (glomerular and alveolar BM), but also the relative concentration of the different BM components can vary (8, 9). A unique deviation of the usual BM structure and composition occurs in the epithelial BM of the human cornea. In contrast to the peripheral/conjunctival epithelial BM, the central corneal epithelial BM lacks immunoreactivity with antibodies to type IV collagen. Ultrastructurally a continuous lamina densa, which consists largely of type IV collagen molecules (10, 11), is absent. However, it can be argued that an epithelial/stromal interface without type IV collagen should not be called a BM. What goes on in the cornea? Are the epithelial cells directly anchored to the stromal collagen or does the stromal collagen locally replace the function of type IV collagen? One puzzling enigma, which is not discussed in the previous chapters, complicates this issue even further. The biochemical characterization of the target antigens of monoclonal antibodies (MA) 1052, 1053 and 1087, convincingly shows that they are reactive with the globular NC1 domain of type IV collagen. As one would expect, the central epithelial corneal BM does not react with MA 1087, but surprisingly MA 1052 and 1053 do react with this BM. A speculative explanation for these findings could be that the central corneal BM contains an altered type IV collagen which is not electrondense as in a lamina densa and does not react with most antibodies against type IV collagen.

Although a continuous BM surrounds some mesenchymal cells, such as fat, Schwann and muscle cells, other mesenchymal cells like myofibroblasts display discontinuous pericellular deposits of BM-like material. The BM patches are often irregularly shaped which suggests that some mechanisms regulating the assembly of BM components into a normal continuous BM are lacking. These findings, together with the heterogeneous BM immunoreactivity observed with the MA 1052 and 1053 as well as other findings which illustrate the heterogeneous nature of BM (12, 13) lead to the conclusion that the structure and composition of a BM depends upon the tissue in which the BM is situated. The glomerular BM plays a specific role in ultrafiltration, and in contrast the epidermal BM has

an important barrier function, and undoubtedly both are mediated by specific BM components. Furthermore, it is not unlikely that also the supramolecular structure of the common BM components is variable.

8.4 How is the epithelial BM deposited?

The generally accepted concept is that the epithelial BM is produced by the epithelial cells, adherent to this BM (8). However, taking into consideration that the BM is a layered structure with particular components occurring in different layers, as for example type IV collagen in the lamina densa (10, 11) and types IV and VII collagen in anchoring plaques in the mesenchyme (14), the mechanisms which regulate the production and assembly of BM components into a structurally recognizable BM must be rather complex. If epithelial cells produce all these components, newly synthesized molecules have to be transported through the entire BM before they can be deposited into e.g. the anchoring plaques. This is not impossible but it is hard to conceive of the mesenchyme as an inert domain as far as the BM is concerned.

In chapter 6 we could demonstrate that the epithelial BM of human tumor cells, xenografted into athymic animals, but also in the normal rat epidermis, is (partly) derived from mesenchymal cells. The mesenchymal cell, which is most likely responsible for the production and deposition of mesenchymal BM components, is the myofibroblast. In chapter 5, we demonstrated that myofibroblasts can produce type IV collagen and laminin both in vivo and in vitro. Fibroblasts did not appear to do this, at least according to the techniques used, which appears to contradict the results of other investigators (15, 16), who showed that normal skin fibroblasts and embryonal fibroblasts may contain type IV collagen mRNA and produce type IV collagen. The nature of the myofibroblast is still incompletely resolved. Whether or not the umbilical cord myofibroblasts are comparable with the myofibroblasts which occur in healing wounds or desmoplastic reactions remains to be established. In normal resting tissue this cell type might contribute to the production and maintenance of the epithelial BM, but it cannot be excluded that these cells are rare in normal tissues, and that in this situation the epithelial BM is entirely an epithelial product. In desmoplasia and tissue repair they might be induced by the growth factors which sustain the neoplastic or reparative proliferation, transforming from fibroblasts into myofibroblasts, which then also contribute to the deposition of BM.

If the epithelial BM is the result of assembly of BM components produced by both epithelial cells and myofibroblasts the question arises which mechanisms in the interaction between epithelium and mesenchyme regulate BM deposition. In vitro studies have almost invariably shown that BM are not deposited adjacent to cells in tissue culture, which do produce BM components, in or on type I collagen cultured with or without mesenchymal cells and with or without additives in the culture medium (chapter 7). Our experiments have therefore failed to identify a trigger, needed for the assembly of components produced in vitro into a BM. Strikingly, xenotransplantation of collagen lattices with epithelial

cells results within 2-3 days in linear BM formation at the interface between the epithelial cells and the type I collagen lattice (17). These findings illustrate that in vitro the cellular, extracellular matrix or molecular components which are necessary for the assembly of BM are missing, because in vivo the formation of a BM is rapidly induced. Knowledge of these regulatory mechanisms would shed new light on aspects of normal tissue development during embryology and tissue homeostasis. Such knowledge might also tell us why do tumor cells differ in their tendency to deposit a BM at the stromal/epithelial interface, which appears to be a general reflection of the biological behaviour of the tumour cells as has been shown in colonic carcinoma (18).

8.5 Future perspective

The three questions posed in this general discussion have largely remained unanswered. We do not know whether or not more BM components exist, if so how many, what the molecules would look like and what their function might be. The interface between the central corneal epithelium and the underlying stroma and the nature of the myofibroblast remain intriguing puzzles. The origin of the epithelial BM and the regulation of its deposition can still be debated. These puzzles should be solved. Not only because questions always cry for an answer but because these answers would complete our basic insight in the composition, structure and function of the BM. This extracellular structure plays a central role in a variety of embryological, physiological and pathological phenomena, which will only be completely understood when the BM has unveiled most of its secrets.

8.6 References

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Chapter 9

Addendum

9.1 Immunization and monoclonal antibody production

9.1.1 Immunization with amniotic BM

Stripped amniotic membranes (1.5 cm²) were subcutaneously implanted three times with monthly intervals into the neck of Balb/c mice (TNO, Zeist). After three months the mice were boosted intraperitoneally with minced amniotic membranes, supplemented with 50 µg of a mixture from type IV and V collagen (1) and an 8 M Urea extract of amniotic membranes (2). Three days after final booster injection, the spleen was removed for fusion.

9.1.2 Immunization with glomerular BM

Balb/c mice were immunized with 200 µg freeze dried glomerular BM in Freund's complete adjuvant. The mice were boosted four times with 200 µg freeze dried glomerular BM in Freund's incomplete adjuvant. Three days after the final booster injection the spleen was removed for fusion.

9.1.3 Monoclonal antibody production

A cell suspension of spleen cells was made in RPMI-medium by mincing the spleen through a sieve. Red blood cells were lysed by incubation with 0.155 M NH₄Cl, 0.01 M KHCO₃, 0.1 mM EDTA, pH 7.4 for 2 min. After three washings with RPMI, the cells were mixed (2:1) with Sp2/0 myeloma cells and centrifuged 5 min 1000 rpm. The cell pellet was gently dissolved in 40 % Polyethylene glycol (PEG 4000, Merck, Darmstadt, FRG) and incubated for 2 min 37 °C. The fused cells were gently dissolved in RPMI and centrifuged 5 min 800 rpm. The cell pellet was dissolved in 25 ml Hypoxanthine-Aminopterin-Thymidine (Flow, Amstelslad, Zwanenburg, The Netherlands) supplemented Dulbecco's modified minimum essential Eagle's medium (Flow, Amstelslad, Zwanenburg, The Netherlands) and 20 % fetal calf serum (Boehringer, Mannheim, FRG), and diluted in five 96-wells plates. After 3 and 7 days the medium was changed. Ten days after fusion the supernatants were tested on 4 µm frozen tissue sections as described under immunohistochemistry (chapter 9.2). Positive clones were recloned by limiting dilution until monoclonal, and tested for the presence of one isotype by ELISA using a mouse isotype kit (Bio-rad, Utrecht, The Netherlands).

9.2 Immunohistochemistry

Frozen sections (4 µm) from human tissues were air-dried 15 min and fixed in acetone (-20 °C, 20 min). Paraffin sections (4 µm) of 4% phosphate buffered paraformaldehyde fixed tissues were deparaffinized with xylol, hydrated and washed with PBS. Successively, frozen and paraffin sections were incubated (20 min) with methanol and 0.3% H₂O₂ to inactivate endogenous peroxidase reactivity, and washed 3 times 5 min with PBS. The sections were

incubated with the primary antibody (1 hr, room temperature (RT)). After washing with PBS (3 times 5 min) the sections were incubated (1 hr, RT) with a conjugated second layer, either peroxidase conjugated rabbit anti mouse IgG (Dakopatts, Glostrup, Denmark) or fluoresceine isothiocyanate conjugated rabbit anti mouse IgG (Dakopatts, Glostrup, Denmark), for monoclonal antibodies or peroxidase conjugated goat anti rabbit IgG (Nordic, Tilburg, The Netherlands) for polyclonal rabbit antisera. All antibody dilutions were prepared with PBS containing 1% (w/v) bovine serum albumin (BSA, RIA grade, Sigma, St. Louis, USA). After washing in PBS (3 times 5 min), peroxidase activity was visualized by incubation with 3,3' diaminobenzidine (DAB, Serva, Heidelberg, FRG) (0,5 mg/ml in 50 mM Tris pH 7,6 and 0,002% H₂O₂) for 7 min in the dark. Finally, the sections were washed in PBS and lightly counterstained with hematoxylin.

9.3 Tissue pretreatment

In order to expose the antigenic sites not accessible due to fixation and paraffin incubation, paraffin tissue sections were pretreated with pronase (Boehringer, Mannheim, FRG) 400 µg/ml in 50 mM TRIS-HCl buffer, pH 8.0, for 30 minutes at 37 °C (1) when monoclonal antibodies were applied, or with pepsin (Boehringer, Mannheim, FRG) 1 mg/ml in 0.01 N HCl, 30 minutes at RT for the polyclonal antisera after deparaffinization and prior to inactivation of endogenous peroxidase reactivity.

9.4 Antibodies

The source and specifications of the antibodies used in this thesis are listed in table 1.

9.5 Electrophoresis and Western blotting

Freeze dried proteins were dissolved in sample buffer (0.01 M Tris, 0.001 M EDTA, 1 % SDS, pH 8.0). Reduction of the proteins was performed by adding 5 % β-mercaptoethanol and heating the sample during 5 min at 100 °C. The proteins were run on a 10 % acrylamide/2.7 % crosslinking separation gel and 5 % acrylamide/2.7 % cross-linking stacking gel or on a 5 % acrylamide/ 2.7 % crosslinking separation gel and 3 % acrylamide/ 2.7 % cross-linking stacking gel. The proteins were run 45 min (12) in a mini-Protean II electrophoresis cell (Bio-rad, Utrecht, The Netherlands). After electrophoresis the gels were incubated 3 times 5 min with blotting buffer (25 mM Tris, 192 mM Glycin, 20 % methanol (v/v), pH 8.3) to get rid of most of the SDS, and subsequently blotted (13) to nitrocellulose paper (Schleicher & Schüll, Dassel, FRG) using a mini-transblot electrophoretic transfer cell (Bio-rad, Utrecht, The Netherlands). Reference lanes with molecu-

Table 1. Specification of the antibodies used in this study.

code	source	antigen	specifications
1042	ma	human pepsin cleaved col IV	reacts with human and rabbit BM, (IgG2b), reacts with the triple helix of type IV collagen (1)
1043	ma	human pepsin cleaved col IV	human specific, (IgG1) reacts with the triple helix of type IV collagen (1)
1087	ma	human glomerular BM	human specific, (IgG2b) reacts with the NC-1 domain of type IV collagen (3)
1052	ma	human amniotic membranes	human specific, (IgG1), reacts with the NC-1 domain of type IV collagen, heterogeneous staining pattern (3)
1053	ma	human amniotic membranes	human specific, (IgG1), reacts with the NC-1 domain of type IV collagen, heterogeneous staining pattern (3)
1065	ma	human amniotic membranes	human specific, (IgG2b), heterogeneous staining pattern (3)
4E10	ma	human placental laminin	reacts on immunoblot with 200-220 kD laminin bands (4)
NP-76	ma	human type VII collagen	directed against the carboxyterminal domain of type VII collagen (5)
MAB458	ma	bovine HSPG (protein core)	reacts with bovine, human, rabbit and dog BM (Chemicon International Inc., U.S.A.)
pa col IV	pa	human pepsin cleaved col IV	reacts with human, rabbit, mouse, rat, dog BM, directed against epitopes in the triple helix of type IV collagen (1)
20451	pa	mouse EHS col IV	reactive with mouse and rat BM, no immunoreactivity with human BM (6)
pa laminin	pa	mouse EHS laminin	reacts on immunoblot with 200-220 kD band no reaction with type I, III and IV collagen on antigen spot test
57 E/6	pa	rat yolk sac laminin	reacts on immunoblot with the 200-220 kD laminin bands (4)
pa col I	pa	human pepsin cleaved col I	reacts on immunoblot with the 95, 100 and 200 kD bands of type I collagen
MAS 037C	ma	human plasma fibronectin	reactive with fibroblasts, and sometimes with basement membranes. (Sera Lab, England)
DAKO-VWF	ma	factor VIII	reactive with endothelial cells. (Dakopatts, Denmark)
MDE-II	ma	chicken gizzard desmin	reacts on immunoblot with 53 kD desmin band. Cross-reactive with human desmin. (Organon Teknika, The Netherlands)
PDE	pa	chicken gizzard desmin	reacts on immunoblot with 53 kD desmin band. Cross-reactive with human desmin. (Organon Teknika, The Netherlands)
MVI	ma	calf lens vimentin	reacts on immunoblot with 57 kD vimentin band. (Organon Teknika, The Netherlands)
PVI	pa	calf lens vimentin	reacts on immunoblot with 57 kD vimentin band. Cross-reactive with human vimentin. (Organon Teknika, The Netherlands)
	pa	smooth muscle myosin	myosin from calf aorta, cross-reactive with human myosin (7)
RPN.1169	ma	mouse pan myosin	cross-reactive with human myosin. Reacts with the heavy chain the fast and slow myosin isoenzymes. (Amersham, England)
HHF 35	ma	α and γ muscle actin	reactive with 42 kD isotypes (α and γ) of skeletal, cardiac and smooth muscle cells (8, 9)
	ma	α smooth muscle actin	monoclonal antibody against a synthetic decapeptide, sequence of the NH ₂ -terminal peptide of α -smooth muscle actin (10)
R11D10	ma	human cardiac myosin	reactive with the heavy chain (type I) slow myosin isolated from left ventricle (11)

ma = monoclonal antibodies, pa = polyclonal antibodies, col IV = type IV collagen, col I = type I collagen. BM = basement membrane(s), EHS = Engelbreth-Holm-Swarm tumor, HSPG = heparan sulphate proteoglycan.

lar weight standards (Bio-Rad, Utrecht, The Netherlands) were washed with PBS and 0.3 % Tween-20 and stained with Aurodye 0.2 ml/cm² (Janssen, Life Sciences, Olen, Belgium).

The nitrocellulose paper for immunostaining was treated either with PBS and 1 % BSA or PBS and 0.3 % Tween-20, and incubated with antibodies as described under immunohistochemistry (chapter 9.2).

9.6 Northern blotting

Cultured cells were isolated by trypsin digestion (3 min at 37 °C with 0.25 % trypsin (Difco Laboratories, Detroit, USA), in 0.1 M NaCl, 0.01 M Tri-sodium citrate, 0.0015 % phenol red, pH 7.8), washing with PBS and centrifugation for 10 min at 500 g. Cytoplasmic RNA was isolated by resuspending the cell pellet 1:2 (v/v) in 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 150 mM NaCl, 0.65% NP40 and incubation for 5 min at 0 °C (14). This solution was centrifuged for 5 min at 5000 g. The supernatant was extracted four times with an equal volume of phenol/chloroform/isoamylalcohol 25:24:1 (v/v/v) and 0.2% (v/v) 8-hydroxyquinoline (in 10 mM NaAc, 1 mM EDTA, 100 mM NaCl pH 5) and centrifuged 5 min 3000 g. The aqueous phase was extracted three times with Chloroform/isoamylalcohol 24:1 (v/v). Finally, the RNA was precipitated overnight 4 °C in 1:2 (v/v) alcohol 96%. The RNA was dissolved in 10 mM Tris-HCl, 1 M EDTA, 100 mM NaCl, 0.5% SDS pH 7.8. 10-20 µg total RNA was separated by electrophoresis in 1% agarose-formaldehyde gels (1 % agarose in 20 mM 3-Mospholonipropanesulfonic acid, 5 mM NaAc, 1 mM EDTA, 6 % formaldehyde) for 22 hr at 1.5 Vcm⁻¹ and blotted overnight onto a Genescreen plus filter (NEN Research Products, Boston, USA) in a buffer consisting of 254 ml 20x SSC (3 M NaCl, 0.3 M Na-citrate pH 7.0) and 46 ml 37% formaldehyde. The blot was air dried and baked for 2 hr at 80 °C. The filter was prehybridized during 16 hr at 42 °C in a solution containing 0.75 M NaCl, 75 mM Na-citrate, 50 mM Na-phosphate, 0.02% (w/v) polyvinyl pyrrolidone, 0.02% (w/v) bovine serum albumin, 0.02% (w/v) ficoll, 250 µg/ml denaturated salmon sperm DNA (Sigma, Detroit, USA) and 50% formamide. Hybridization of RNA-cDNA was carried out for 24 h at 42 °C in the same solution containing the labeled type IV collagen cDNA probe (15). The type IV collagen probe was labeled with the random primer labeling method (16, 17) with ³²P-dCTP (3000 Ci/mM, Amersham, Utrecht, The Netherlands) to a specific activity of at least 5 10⁸ cpm/µg. After hybridization the filter was washed 15 min in 2x SSC, 1% SDS 60 °C, two times 15 min in 1x SSC, 1% SDS 60 °C and 15 min in 0.3x SSC, 1% SDS 65 °C. The filter was dried and autoradiographed using Kodak X-Omat films, for 24-48 hr.

9.7 In situ hybridization

All solutions used in this procedure were treated with 0.1 % diethylpyrocarbonate (DEPC) to inactivate RNase activity. Glass slides were coated with 0.15% (w/v) chrome alum and 0.43% (w/v) gelatin. 4 μ m frozen sections were mounted on RNase free coated slides, fixed 5 min with freshly prepared 4% paraformaldehyde and washed three times 5 min with PBS. Subsequently the slides were baked at 45 °C for 2 h. The slides were washed 3 min in PBS (0.1 M, pH 7.2) at RT, treated 15 min with 0.3% Triton X-100 in PBS and washed two times 3 min with PBS. Furthermore, the sections were incubated for 20 min at 37 °C with 1 μ g/ml proteinase K (Sigma, Detroit, USA) in 0.1 M Tris (pH 8.0), 50 mM EDTA, fixed again (5 min RT) with 4% buffered paraformaldehyde, washed two times 3 min with PBS and treated (10 min, RT) with 0.25% acetic anhydride in 0.1 M triethanolamine pH 8.0 and furthermore incubated (at least 10 min, 37 °C) with 50% formamide in 2x SSC (2x SSC = 0.3 M NaCl, 0.03 M trisodium citrate pH 7.0). ³⁵S-dCTP labeled type IV collagen probe (15) was prepared according to the random primed labeling method using a Boehringer labeling kit (16, 17). 15 ml probe/hybridization mixture containing 300.000 cpm was applied to each section. The sections were subsequently covered with 22x22 mm siliconized coverslips and sealed with rubber cement. Hybridization mixture contained yeast t-RNA (500 μ g/ml), salmon sperm DNA (500 μ g/ml), 50% deionized formamide, 10 mM Tris-HCl pH 7.4, 0.3 M NaCl, 1 mM EDTA, 2x Denhardt's mixture (0.04 % bovine serum albumin, 0.04 % Ficoll 400, 0.02 % polyvinylpyrrolidone), 10% Dextran sulphate (Pharmacia, Uppsala, Sweden), 30 mM DTT. After 12-16 h incubation at 50 °C, the coverslips were removed and the slides were washed four times 30 min RT with 4x SSC, three times 10 min at 37 °C with 2x SSC and rinsed three times 10 min with 0.1x SSC. Following these steps the slides were dehydrated in graded series of ethanol, air dried and coated with Kodak NTB-2 nuclear track emulsion and exposed for varying periods of time. The exposed slides were developed in 1:1 Kodak D-19 developer (4 min, 13-15 °C), fixed, and counterstained with hematoxylin, dehydrated and mounted. Parallel sections were hybridized with an ³⁵S labeled empty plasmid as a negative control.

9.8 Type I collagen lattices

9.8.1 Isolation of human type I collagen

Human type I collagen was prepared from human placenta by limited pepsin digestion and salt precipitation as described by Havenith et al. (1, 18). Briefly, a placenta was washed 3 times 3 hr with water, 4 times 6 hr with 1 M NaCl, 50 mM Tris-HCl pH 7.5 and 4 times 6 hr with 0.5 M HAc. The placenta was minced with a meat grinder and digested 24 hr with pepsin (Boehringer, Mannheim, FRG) enzyme/substrate ratio 1:400 (wet weight) dissolved in 1.5 M HAc pH 2.8. The undigested material was separated by centrifugation 30 min 7000

g. The digest was precipitated with 0.7 M NaCl (by adding solid NaCl). The precipitate was dissolved again in 1.5 M HAc and precipitated once more with 0.7 M NaCl. This precipitate was dialyzed against 0.5% HAc and freeze dried.

9.8.2 Isolation of rat type I collagen

Rat type I collagen was prepared by dissecting the four tendon bundles from the rat tail (19). The bundles were washed 30 min with 70% ethanol and incubated with PBS. Subsequently 1 g of stripped and washed tendons was incubated with 100 ml 0.1% HAc for 7 days at 4 °C. The collagen mixture was clarified by centrifugation at 10000 g and freeze dried.

9.8.3 Preparation of type I collagen lattices

Type I collagen lattices (20, 21) were prepared by mixing a cold stock solution of type I collagen (4 mg/ml in 0.1 % HAc) with culture medium and 0.1 N NaOH to neutralize the pH. The end concentration of type I collagen was 0.7, 1 or 2 mg/ml. Reaggregation took place as soon as the solution was neutralized and brought to 37 °C.

9.9 References

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Summary

This thesis focusses on aspects of the heterogeneity of basement membranes (BM). Chapter 1 provides a brief introduction on the structure and composition of normal and pathological BM. The BM components which occur in almost all BM are briefly described as well as the components with a more limited tissue distribution. Furthermore the aim of the studies described in this thesis is outlined.

In chapter 2 the development of BM reactive monoclonal antibodies (MA), by immunization of mice with intact denuded BM is described. MA 1052 and 1053 reacted with the BM of the epidermis and epidermal adnexa and furthermore in the lung with the BM of the alveolar epithelium. In the gastrointestinal tract only the superficial part of the epithelial BM reacted. MA 1065 showed immunoreactivity with the epidermal and adnexal BM in the skin and the epithelial BM of trachea and oesophagus, and furthermore showed pericellular staining of the basal keratinocytes and basal corneal epithelial cells. MA 1087, raised against human glomerular BM, showed immunoreactivity with all BM, except the central epithelial BM in the cornea. Furthermore the susceptibility of the target epitopes of these MA for various enzymes, and the localization of the epitopes in the different layers of the BM was tested, the latter by chemical splitting of human skin. The results indicate that the epitopes of MA 1052, 1053, 1065 and 1087 are susceptible for proteases, but not degraded by bacterial collagenase.

In chapter 3 the epitopes of MA 1052, 1053 and 1087 are characterized. Despite the differences observed by immunohistochemical evaluation of the tissue distribution patterns of these antibodies described in chapter 2, all three antibodies reacted with the same bands on western blots of isolated and collagenase digested glomerular and amniotic BM. Comparing the molecular weight of these bands with the stained bands on immunoblots of sera from patients with anti-glomerular BM autoantibodies, directed against the NC1 domain of type IV collagen, identical patterns of immunoreactivity were found. From these findings we conclude that all three monoclonal antibodies are reactive with epitopes localized in the non-collagenous (NC1) domain of type IV collagen.

An example of BM heterogeneity in connection with one type of epithelium is described in chapter 4. We found that central human epithelial corneal BM lacks immunoreactivity for type IV collagen, using various anti-type IV collagen antibodies. In similar experiments laminin was found uniformly distributed in the entire corneal epithelial BM. By electron-microscopy the central corneal region did not show a characteristic epithelial BM. Several explanations are considered for the apparent lack of type IV collagen immunoreactivity: 1. Type IV collagen is totally absent and a different structural component is deposited or Bowman's membrane takes over type IV collagen function; 2. Type IV collagen determinants are masked by glycoproteins or proteoglycans; 3. The arrangement of BM components in a supramolecular structure is altered resulting in loss of type IV collagen determinants. If type IV collagen is indeed absent this could be due to the high level of differentiation of central corneal epithelium, which is generated in the limbal proliferation

zone, assuming that highly differentiated epithelium can not produce a BM. Alternatively, the acellular Bowman's membrane might lack triggers to induce its production by the epithelial cells. It is concluded that central human corneal epithelial BM is the only BM which lacks immunoreactivity with antibodies to type IV collagen, and therefore the basic structure of this BM might be different from that of other BM.

In chapter 5 we describe the isolation and cultivation of umbilical cord stromal cells. The stromal cell by immunohistochemistry appears to combine characteristics of muscle cells, in containing desmin, smooth muscle actin and myosin and being enveloped by BM components, and of fibroblasts, in being reactive with fibronectin. Ultrastructurally, these cells have the same characteristics as myofibroblasts in granulation tissue or in healing wounds. They contain fibroblast-like endoplasmic reticulum, peripheral parallel cytoplasmatic filaments with dense body condensations and discontinuous extracellular BM-like material. Therefore, we conclude that the stromal cells of the umbilical cord stroma are myofibroblasts. Myofibroblasts appear to be the only cell type which produce ample amounts of BM material which is not deposited into a continuous BM.

In chapter 6 experiments are described with human colonic tumor cell lines (5583-E, HT-29), which do not produce immunoreactive BM type IV collagen *in vitro*. These cells were xenografted into nude rats to generate epithelial neoplasms. By immunohistochemistry, no human but only rat type IV collagen containing BM were observed at the transition of tumor cells and host stroma. Two other tumor cell lines (WISH, KB), showed BM structures *in vivo* which stained both with human and host specific anti-type IV collagen antibodies. These findings were in agreement with results of *in situ* hybridization experiments using a type IV collagen c-DNA probe. In the xenografts of 5583-E and HT-29, hybridization was observed exclusively with the rat mesenchyme between the tumor cells, whereas xenografts of WISH and KB showed a hybridization reaction with the tumor cells and rat stromal components. Furthermore in rat epidermis hybridization was observed with basal keratinocytes and with the stroma adjacent to the epithelial BM. From these results we conclude that in xenografts of human cancer cells but also in normal epithelia, stromal cells contribute to the deposition of a BM.

In chapter 7 experiments are described which attempted to screen human normal and tumor cells in their capability to produce BM components *in vitro*. Cells were grown on gelatin coated glass slides or on denuded amniotic BM or cytocentrifuged on glass slides and stained with antibodies to BM components. Culture supernatants were precipitated and subsequently electrophoresed and immunoblotted using antibodies to BM components. Cytoplasmic type IV collagen mRNA was determined by Northern blotting. Furthermore, the role of artificial extracellular matrix was investigated in its ability to influence the production of BM components *in vitro*. Only a few cell lines (KB, WISH and myofibroblasts) were still capable to produce BM components *in vitro*.

In chapter 8, the results of the studies are discussed in a broader context.

Finally, in chapter 9 some of the techniques used in the experiments reported in this thesis are extensively described.

So far BM research has primarily focussed on the components which can be observed in nearly all BM. Our experiments show that the macromolecular structure of the BM and even the molecular structure of individual BM components may vary depending on the cell or tissue type.

We furthermore show that, in contrast to the generally accepted concept that "epithelial" BM are produced by epithelial cells, mesenchymal cells do contribute to the production and deposition of BM components at the boundary of epithelium and underlying stroma, which lead to the formation of the epithelial/mesenchymal BM.

Samenvatting

In dit proefschrift worden enkele aspecten van basale membraan heterogeniteit (BM) belicht.

In hoofdstuk 1 wordt de structuur en samenstelling van de normale en pathologisch afwijkende BM beschreven. De BM componenten worden ingedeeld in bestanddelen die in alle BM worden waargenomen en componenten die een gelimiteerde BM distributie hebben. In het laatste gedeelte van dit hoofdstuk wordt de vraagstelling van dit proefschrift geschetst.

In hoofdstuk 2 wordt de ontwikkeling van monoclonale antilichamen (MA) beschreven, door muizen te immuniseren met intacte BM. MA 1052 en 1053 reageren met de BM van huid, talg- en zweetklieren en haarschachten van de huid en verder in de long met de BM van het alveolaire epitheel. In de maag-darm tractus reageren deze monoclonalen alleen met de BM aan de top van de villi of crypten. MA 1065, daarentegen, reageert alleen met de BM van de huid, klieren en haarschachten van de huid en met de epitheliale BM van slokdarm en luchtpijp. Verder reageert MA 1065 ook pericellulair met basale huidcellen en basale cornea epitheelcellen. MA 1087, opgewekt door immunisatie met glomerulaire BM, reageert met alle BM, met uitzondering van de centrale epitheliale BM in de cornea. Vervolgens wordt de gevoeligheid van de epitopen van deze MA voor verschillende enzymen beschreven. De epitopen van MA 1052, 1053 en 1065 zijn gevoelig voor proteases, maar blijven intact na collagenase-digestie. De localisatie van de epitopen in de verschillende lagen van de BM, gebeurde na chemische splitsing in de BM. Stukjes humane huid worden geïncubeerd met een zoutoplossing, waarna de opperhuid van de lederhuid kan worden gescheiden, waarbij de splitsing plaats vindt binnen de BM. De verschillende lagen worden afzonderlijk aangekleurd met de MA. De epitopen van MA 1052 en 1053 komen voor in het gedeelte van de BM dat na splitsing vastzit aan het stromale deel van de huid. Het epitoom van MA 1065 wordt gelocaliseerd in het BM-gedeelte, dat na splitsing direct tegen de epitheel cellen aanzit. Bovendien wordt dit epitoom waargenomen rondom de basale epitheel cellen.

In hoofdstuk 3 worden de epitopen van MA 1052, 1053 en 1087 nader gekarakteriseerd. In tegenstelling tot de verschillen die qua aankleuringspatroon tussen deze MA wordt waargenomen (hoofdstuk 2), reageren deze drie antilichamen met dezelfde eiwitbanden op western blots, van collagenase gedigesteerde glomerulaire en amnion BM. Identieke aankleuringspatronen worden waargenomen, wanneer de molecuul gewichten van deze banden op western blot worden vergeleken met de aankleuringspatronen van sera van patiënten met anti-glomerulaire BM autoantilichamen, gericht tegen het NC1 domein van type IV collageen. Uit deze bevindingen kunnen we concluderen dat de MA 1052, 1053 en 1087 gericht zijn tegen epitopen die zich binnen het NC1-domein van type IV collageen bevinden. De moleculaire structuur van dit NC1-domein verschilt afhankelijk van weefsel of cel type.

Een voorbeeld van BM heterogeniteit binnen één type epitheel wordt beschreven in hoofdstuk 4. De epitheliale BM van het centrale deel van de cornea bevat geen type IV collageen, terwijl andere BM componenten, zoals laminine en heparan sulfaat proteoglycaan, over de totale lengte van de cornea wordt waargenomen. Ultrastructureel wordt in het centrale gedeelte van de cornea geen normale continue epitheliale BM waargenomen. Mogelijke verklaringen voor de afwezigheid van type IV collageen immunoreactiviteit kunnen zijn:

1. Type IV collageen is totaal afwezig terwijl een andere structurele component of de membraan van Bowman deze structurele functie van type IV collageen overneemt.
2. Type IV collageen epitopen worden gemaskeerd door andere BM componenten zoals glycoproteïnes of proteoglycanen.
3. De supramoleculaire structuur van BM componenten is veranderd waardoor de type IV collageen epitopen verloren gaan. De afwezigheid van type IV collageen kan worden verklaard door de hogere differentiatie graad van het centrale cornea epitheel t.o.v. het epitheel in het perifere gedeelte. Hoger gedifferentieerde cellen zijn mogelijk niet meer in staat type IV collageen te produceren. Een andere mogelijkheid is, dat de acellulaire en avasculaire membraan van Bowman bepaalde prikkels mist, die nodig zijn voor het epitheel om type IV collageen te produceren.

Concluderend kunnen we stellen dat de centrale cornea epitheliale BM de enige BM is die type IV collageen mist. De basale structuur van deze BM is waarschijnlijk verschillend van andere BM.

In hoofdstuk 5 wordt de isolatie en het kweken van navelstreng stroma cellen beschreven. Deze stroma cel bevat immunohistochemische karakteristieken zowel van gladde spiercellen (desmine, glad spierweefsel actine, myosine), als van fibroblasten (fibronectine). Ultrastructureel bevatten deze cellen dezelfde karakteristieken als myofibroblasten, die o.a. voorkomen bij de wondgenezing. Deze cellen bevatten een fibroblast-achtig gedilateerd endoplasmatisch reticulum, perifere parallelle cytoplasmatische filamenten met focale condensaties, en discontinuë extracellulaire BM deposities. Deze bevindingen suggereren dat de stromale cellen van de navelstreng myofibroblasten zijn. De myofibroblast is het enige cel type dat behoorlijke hoeveelheden BM componenten maakt, maar deze niet in een continue BM structuur afzetten.

In hoofdstuk 6 worden experimenten beschreven met humane colon tumor cellijnen (5583-E, HT-29), die geen type IV collageen maken *in vitro*. Deze cellen werden ingespoten in naakte ratten om epitheliale tumoren te genereren. Met behulp van immunohistochemie wordt geen humane-, maar alleen een ratte-type IV collageen bevattende BM waargenomen bij de overgang van humane tumor cellen en het omliggende gastheer (ratte) stroma. Twee andere tumor cellijnen (WISH, KB), gaven in naakte ratten BM structuren te zien die zowel met humaan als met gastheer specifieke anti-type IV collageen antilichamen reageren. Deze bevindingen zijn in overeenstemming met de resultaten van *in situ* hybridisatie experimenten waarbij gebruik werd gemaakt van een type IV collageen cDNA probe. In de xenografts van 5583 en HT-29 werd alleen hybridisatie waargenomen met cellen in het rattestroma, terwijl xenografts van WISH en

KB, zowel met de tumorcellen als met de cellen in het rattestroma hybridisatie te zien gaven. Verder werd in ratte-epidermis hybridisatie waargenomen zowel in de basale keratinocyten als in stroma cellen aangrenzend aan de epitheliale BM. Uit deze resultaten kan worden geconcludeerd dat in xenografts van humane tumorcellen, maar ook in normale epithelia, stromale cellen een rol spelen bij de depositie van de BM.

In hoofdstuk 7 worden experimenten beschreven waarin wordt getracht de productie van BM componenten *in vitro* te screenen. Cellen werden gekweekt op gelatine gecoate object-glaasjes, op gedenudeerde amnion BM of gecentrifugeerd op glaasjes en vervolgens aangekleurd met de anti-BM antilichamen. Supernatant van deze celkweken werd geprecipiteerd en vervolgens geëlectroforeerd en geblot naar nitrocellulose papier en aangekleurd met de antilichamen gericht tegen BM componenten. Cytoplasmatisch type IV collageen mRNA werd bepaald d.m.v. northern blotting. Verder werd de rol van een artificiële extracellulaire matrix getest op de beïnvloeding van de productie van BM componenten *in vitro*. Slechts een aantal van de geteste tumor cellijnen (WISH, KB, myofibroblasten) waren *in vitro* nog in staat om BM componenten te produceren.

In hoofdstuk 8 worden de resultaten van deze studies belicht tegen een bredere achtergrond. Tevens worden enkele suggesties voor verder onderzoek in de nabije toekomst besproken.

Ten slotte worden in hoofdstuk 9 enkele technieken uitgebreider beschreven.

Tot dusverre is veel onderzoek verricht aan componenten die in alle BM voorkomen. In dit proefschrift wordt echter beschreven, dat de macromoleculaire samenstelling en zelfs de moleculaire structuur van afzonderlijke componenten kunnen verschillen, afhankelijk van het weefsel of de cel waarin deze bestanddelen voorkomen.

In dit onderzoek wordt tevens beschreven, dat in tegenstelling tot de algemeen geaccepteerde opvatting, namelijk, dat de epitheliale BM enkel worden geproduceerd door de aangrenzende epitheelcellen, óók de stromale/mesenchymale cellen een bijdrage leveren bij de tot stand koming van de "epitheliale-mesenchymale" BM.

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CURRICULUM VITAE

J.P.M. Cleutjens werd op 18 juni 1958 geboren in Oirsbeek. In 1977, na het behalen van het eindexamen Atheneum B aan het Sint Jans-college te Hoensbroek, begon hij zijn studie biologie aan de Landbouwhogeschool (nu Landbouwniversiteit) te Wageningen. Op 10 juli 1981 werd het candidaatsexamen biologie behaald. Gedurende de studie heeft hij meerdere malen studenten begeleid tijdens de dierkunde practica voor eerste en tweede jaars biologie en N-20 studenten. Tijdens de studie werd hij opgeroepen onder 's lands wapenrok. Zijn militaire loopbaan startte op 7 maart 1984. Hij werd geplaatst als toegevoegd S2, bij de staf van de 13^e Pantserbrigade te Oirschot. Vanaf 5 juni 1985 werd hem buitengewoon verlof buiten bezwaar rijksschatkist verleend. Op 28 mei 1985 werd het doctoraal examen in de landbouwwetenschappen studierichting biologie behaald met als hoofdvakken celbiologie en dierfysiologie en als bijvak toxicologie. Op 1 juni 1985 werd een begin gemaakt met een promotieonderzoek aan de vakgroep Pathologie aan de Rijksuniversiteit Limburg, getiteld: "Basement membranes in cancer". Dit onderzoek werd mogelijk gemaakt door de financiële steun van het Koningin Wilhelmina Fonds (RUL 85-2).