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COLLAGEN XVII AND PATHOMECHANISMS OF JUNCTIONAL EPIDERMOLYSIS BULLOSA AND GESTATIONAL PEMPHIGOID

FACULTY OF MEDICINE,
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COLLAGEN XVII AND PATHOMECHANISMS OF JUNCTIONAL EPIDERMOLYSIS BULLOSA AND GESTATIONAL PEMPHIGOID

Academic dissertation to be presented, with the assent of the Faculty of Medicine of the University of Oulu, for public defence in Auditorium 5 of Oulu University Hospital, on April 18th, 2008, at 12 noon

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Abstract

Transmembrane collagen XVII (BP180) is a structural component of hemidesmosomes that connects the two layers of skin. Collagen XVII is associated with both autoimmune and inherited bullous skin diseases. Mutations in collagen XVII gene cause junctional epidermolysis bullosa, and in the diseases of the pemphigoid group autoantibodies target collagen XVII. In this work, collagen XVII was studied in both junctional epidermolysis bullosa and gestational pemphigoid.

Two novel glycine substitution mutations were found in the largest collagenous domain of collagen XVII. Analysis of recombinantly produced mutated proteins showed that these novel mutations and previously described glycine substitution mutations decrease the thermal stability of collagen XVII ectodomain. In addition, these mutations were found to cause intracellular accumulation of the mutated proteins and affect the post-translational modifications of collagen XVII. Meanwhile, an in-frame deletion of nine amino acids had no effect on the thermal stability or secretion of the collagen XVII ectodomain.

Gestational pemphigoid autoantigen collagen XVII has been mainly studied in the skin, and its expression and function during pregnancy are so far largely unknown. For the first time, collagen XVII was shown to be expressed by cytotrophoblasts of the first trimester human placenta and by cultured cytotrophoblasts. Transmigration assay of cytotrophoblasts indicated that collagen XVII promotes trophoblast invasion, and may thus have a role in placental formation. In addition, significant amounts of *in vivo* produced collagen XVII were found in the amniotic fluid throughout pregnancy. Collagen XVII expression was also observed in hemidesmosomes of amniotic membranes and in cells cultured from amniotic fluid. These findings suggest that collagen XVII could have a function, albeit so far unknown, during pregnancy.

Keywords: basement membrane, blistering skin disease, BP180, collagen, epidermolysis bullosa, pemphigoid gestationis

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Abbreviations

BP	bullous pemphigoid
BSA	bovine serum albumin
BP180	bullous pemphigoid antigen 180 kD
BP230	bullous pemphigoid antigen 230 kD
C-	carboxy-
cDNA	complementary DNA
COL	collagenous domain
cRNA	complementary RNA
DEAE	diethylaminoethyl cellulose
DEB	dystrophic epidermolysis bullosa
EB	epidermolysis bullosa
EBS	epidermolysis bullosa simplex
H-JEB	Herlitz-type junctional epidermolysis bullosa
HLA	human leukocyte antigen
JEB	junctional epidermolysis bullosa
kb	kilo base
kD	kilo Dalton
LAD	linear IgA dermatosis
mRNA	messenger RNA
N-	amino-
NC	non-collagenous domain
nH-JEB	non-Herlitz-type junctional epidermolysis bullosa
PBS	phosphate buffered saline
PG	gestational pemphigoid
PCR	polymerase chain reaction
PTC	premature termination codon
RT	room temperature
SCC	squamous cell carcinoma
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
X	any amino acid (in Gly-X-Y)
Y	any amino acid (in Gly-X-Y)

List of original articles

This thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I Väisänen L*, Has C, Franzke C, Hurskainen T, Tuomi M-L, Bruckner-Tuderman L & Tasanen K (2005) Molecular mechanisms of junctional epidermolysis bullosa: Col15 domain mutations decrease the thermal stability of collagen XVII. J Invest Dermatol 125: 1112-1118.

II Huilaja L, Hurskainen T, Autio-Harmainen H, Hofmann SC, Sormunen R, Räsänen J, Ilves M, Franzke CW, Bruckner-Tuderman L & Tasanen K (2008) Pemphigoid gestationis autoantigen, transmembrane collagen XVII, promotes the migration of cytotrophoblastic cells of placenta and is a structural component of fetal membranes. Matrix Biol 27:190-200.

III Huilaja L, Hurskainen T, Autio-Harmainen H, Sormunen R, Tu H, Hofmann SC, Pihlajaniemi T, Bruckner-Tuderman L & Tasanen K. Glycine substitution mutations cause intracellular accumulation of collagen XVII and affect the post-translational modifications. Manuscript.

*Huilaja née Väisänen

Contents

1 Introduction

The skin, which is both the biggest and the largest organ of the human body, is more than just a cover. It protects the body against micro-organisms, ultraviolet radiation, loss of water and mechanical stress. Besides, it has multiple functions ranging from immunology to vitamin synthesis and inter-human communication. To fulfil all these tasks the skin has a highly specified structure within its two layers, epidermis and dermis. However, the structure and functions of skin may be deteriorated by autoimmune or inherited skin diseases.

Transmembrane collagen XVII (BP180) is a structural component of hemidesmosomes that maintains the integrity of the epidermis and dermis. In autoimmune diseases of the pemphigoid group autoantibodies are mainly targeted against collagen XVII, resulting in detachment of basal keratinocytes and skin blistering. Similarly, mutations in collagen XVII gene in inherited junctional epidermolysis bullosa lead to synthesis of functionally hampered collagen XVII and cause blistering of the skin.

In this thesis, collagen XVII was studied in both inherited and autoimmune blistering skin diseases. Molecular mechanisms of missense mutations in collagen XVII gene (COL17A1) were studied by using recombinant expression of mutated collagen XVII. As an autoantigen in gestational pemphigoid, the collagen XVII expression pattern in human placenta and foetal membranes was investigated by immunohistochemistry, *in situ* hybridization and immunoelectron microscopy. In addition, the effect of collagen XVII on the placental cell migration was explored in cell culture. Related to these themes, the literature review provides an overview on the structure of skin basement membrane zone and transmembrane collagens as well as blistering skin diseases related to collagen XVII. A general picture of placental development is also addressed due to its importance in the collagen XVII expression studies of this work.

2 Review of the literature

2.1 Cutaneous basement membrane zone

Basement membranes are highly organized, extracellular sheet-like structures at the interface between cells, cell sheets and their surrounding connective tissue. The cutaneous basement membrane zone, also known as the dermo-epidermal junction, connects the basal cells of the epidermis to the underlying dermis (Fig. 1). Electron microscopy analysis shows the basement membrane comprising of two layers, lamina lucida and lamina densa. Lamina lucida is an electron-lucid region next to the plasma membrane. Lamina densa is a thicker, electron dense layer adjoining dermal cells. Most epidermally situated intermediate filaments are inserted into the hemidesmosome, which is further connected with anchoring filaments to the underlying anchoring fibrils within papillary dermis. (Litjens *et al.* 2006, Masunaga 2006, McMillan *et al.* 2003, Tidman & Eady 1985, Yurchenco & Schittny 1990.)

At the molecular level basement membrane is mainly composed of two different networks, one composed of collagen IV and another of laminin isoforms. These networks are linked with fibulin 2, nidogen, fibronectin and a heparan sulphate proteoglycan perlecan, which can also crosslink the laminin and collagen IV networks within the lamina densa. The cutaneous basement membrane zone both separates the distinct parts of skin and assures with its complex structure the stability of the dermo-epidermal junction. It permits both normal and pathological cell migration as well as determines cell polarity, proliferation and differentiation. In addition, the function of the basement membrane zone is crucial in morphogenesis, wound healing and remodelling of the skin. (Burgeson & Christiano 1997, Ghohestani *et al.* 2001, Masunaga 2006, McMillan *et al.* 2003, Timpl 1996, Uitto & Pulkkinen 1996.)

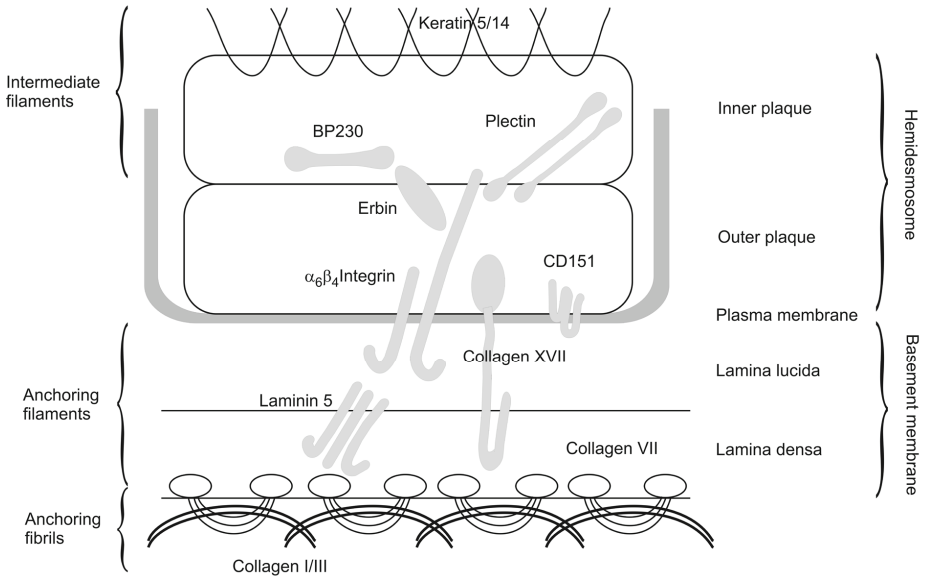

Fig. 1. Epidermal basement membrane zone and hemidesmosomal adhesion complex. (Modified from Aumailley *et al.* 2006, Masunaga 2006, McMillan *et al.* 2003.)

2.1.1 Hemidesmosomal adhesion complex

Hemidesmosomes are small epithelial adhesion complexes by which the basal epithelial cells are connected to the underlying basement membrane. Besides promoting adhesion, they are also involved in signal transduction via $\alpha_6\beta_4$ integrin. In addition, hemidesmosomes are dynamic, with ability to assemble and disassemble (Tsuruta *et al.* 2003). Hemidesmosomes are situated within the plasma membrane on the ventral surface of the basal keratinocytes (Fig. 1). In electron microscopy hemidesmosomes appear as an electron-dense structure composed of an inner and outer plaque and the sub-basal dense plate. In the skin, the inner plaque of the hemidesmosome consists of plectin, BP230 (also known as bullous pemphigoid antigen 1, BPAG1) and erbin, and the outer of $\alpha_6\beta_4$ integrin, collagen XVII (BP180) and tetraspanin molecule CD151 (Favre *et al.* 2001, Sterk *et al.* 2000). (Borradori & Sonnenberg 1999, Hirako & Owaribe 1998, Jones *et al.* 1998, Litjens *et al.* 2006, Nievers *et al.* 1999.)

Keratins 5 and 14 are specifically expressed in the cytoplasm of the epithelial cells (Irvine 2005). These keratins are the structural component of the intermediate filaments to which plakin family proteins, plectin and BP230, associate attaching them to the plasma membrane and forming a stable anchoring complex. Plectin and BP230 are also linked to the transmembrane constituents collagen XVII and $\alpha_6\beta_4$ integrin. Erbin is a possible associating molecule between BP230 and $\alpha_6\beta_4$ integrin. Integrin $\alpha_6\beta_4$ is crucial in the assembly of the hemidesmosomes, and its α_6 subunit interacts with the extracellular domain of collagen XVII (Hopkinson *et al.* 1995, Hopkinson *et al.* 1998). The cytoplasmic domain of collagen XVII links to the β_4 subunit, but this connection only occurs in the presence of plectin (Koster *et al.* 2003). CD151 clusters with $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins, facilitating cell binding. However, CD151 is not necessarily needed in hemidesmosome formation. (Litjens *et al.* 2006, McMillan *et al.* 2003.)

Anchoring filaments are situated beneath the plasma membrane, where the extracellular domain of collagen XVII binds to laminin 5, also referred to as laminin-332 (Aumailley *et al.* 2005). Laminin 5 is a heterotrimer consisting of α_3, β_3 and γ_2 subunits, which form complexes with laminins 6 and 10. These complexes are linked to the collagen IV network via nidogen and fibulin. Laminin 5/6 complex also binds to collagen VII, which provides the hemidesmosomal anchoring complex resistance to frictional forces, whereas the interaction between $\alpha_6\beta_4$ integrin and laminin 5 maintains the stability of the basement membrane zone. (Litjens *et al.* 2006, McMillan *et al.* 2003, Nievers *et al.* 1999.)

Most basal components on the hemidesmosomal complex are anchoring fibrils, which ensure the connection between the epidermal basement membrane and the dermal connective tissue. The anchoring fibrils are trimers of post-translationally modified collagen VII, which are linked either directly to laminin 5 or to the laminin complex via fibulin 2 or fibronectin. Within the upper dermis the anchoring fibrils form semicircular loops, attached at both ends to the lamina densa above (Masunaga 2006). (Bruckner-Tuderman *et al.* 1999, McMillan *et al.* 2003, Varki *et al.* 2007.)

The molecules of the hemidesmosomal anchoring complex bond tightly the keratin cytoskeleton of the basal keratinocyte to the dermal extracellular matrix in a complex chain-like manner. Perturbations in the structures can appear as fragility of the skin and blister formation. (Bruckner-Tuderman 1999, Masunaga 2006, McMillan *et al.* 1998.)

2.2 Biosynthesis of collagens

Collagens are a family of extracellular matrix proteins responsible for tissue integrity and with other important functions as well. They share the typical feature of containing several repeats of the amino acid sequence Gly-X-Y and thus have the characteristic ability of triple-helix folding. (Prockop & Kivirikko 1995.) So far, vertebrates have at least 28 different proteins defined as collagens (Veit *et al.* 2006). Based on subramolecular assembly, the collagens are divided into seven sub-families, the eighth being the family of collagen-like proteins not defined as collagens (Myllyharju & Kivirikko 2004, Ricard-Blum & Ruggiero 2005).

The biosynthesis of collagens has mainly been studied in fibrillar collagens. The fibril-forming collagens are synthesized on the ribosomes of the rough endoplasmic reticulum as propeptides. Propeptides with N- and C-terminal extensions are secreted into the lumen of the endoplasmic reticulum where the signal peptides are removed. Proline and lysine residues of propeptide chains are hydroxylated to 4-hydroxyproline, 3-hydroxyproline and hydroxylysine. All of the enzymes involved in hydroxylation need ascorbic acid as a cofactor. After glycosylation and galactosylation of hydroxylysine, propeptide chains associate through C-propeptides forming a nucleus for triple helical assembly. Inter- and intramolecular disulfide bonds are then formed and a triple helix is propagated from the C- to N-terminal direction in a zipper-like fashion. Procollagen molecules are then secreted to the extracellular space via the Golgi apparatus. Extracellularly, the N- and C-terminal propeptides are cleaved before self-assembly into fibrils. Formation of covalent crosslinks finally stabilizes the molecule. (Mauch & Krieg 1993, Myllyharju & Kivirikko 2001, Myllyharju & Kivirikko 2004.)

The biosynthesis of transmembrane collagens differs from that of fibrillar collagens, since they are anchored to the plasma membrane through the transmembrane domain and their N-terminus is intracellularly situated. This orientation affects the trimer association and triple helix formation. For collagens XIII and XVII it has been shown that their triple helices are propagated from the N- to C-terminus (Areida *et al.* 2001, Snellman *et al.* 2000b). (Myllyharju & Kivirikko 2001, Snellman & Pihlajaniemi 2003.)

The biosynthesis of collagens can be hampered by mutations in collagen genes. The majority of the mutations are single base substitutions converting a codon for glycine to a bulkier residue and thus either preventing the continuing

triple helix from folding totally or causing an interruption in it. The effect of the mutation depends both on the location of the mutation in the triple helix and on the substituting amino acid. It has been shown for procollagens I and III that both glycine substitution mutations and in-frame deletions lead to over-modification of the synthesized protein and thus alter thermal unfolding (Baldwin *et al.* 1989, Pace *et al.* 2001, Tromp *et al.* 1989, Westerhausen *et al.* 1990). Besides affecting the thermal stability, the glycine substitution mutations can also alter the assembly or cause intracellular accumulation of mutated proteins (Brittingham *et al.* 2005, Chan *et al.* 1995, Sawamura *et al.* 2006, Wilson *et al.* 2005). If the structurally altered polypeptide chain can still associate with the normally formed chains this usually causes a more severe effect on the functions of the protein than complete absence of the molecule. This dominant negative effect is due to a mutant chain disturbing the folding or the assembly of collagen triple helix. In addition, mutations that are silent in heterozygous carriers, can cause a severe phenotype when combined, as shown in some patients with DEB (Hammami-Hauasli *et al.* 1997). (Baum & Brodsky 1999, Byers 2001, Myllyharju & Kivirikko 2001, Myllyharju & Kivirikko 2004.)

2.3 Transmembrane collagens

Collagens XIII, XVII, XXIII and XXV form a group of transmembrane collagens. As members of this collagen subfamily they all consist of an intracellular domain, a single membrane spanning domain and a collagenous ectodomain (Fig. 2). Although the length of the intracellular domains differs, they all are type II oriented and proteolytically processed, having dual functions as matrix constituents and cell surface receptors. These transmembrane collagens belong to a broader group of transmembrane proteins in which seven different collagen-like transmembrane proteins, ectodysplasin-A, macrophage scavenger receptors I, II and III, macrophage receptor MARCO (macrophage receptor with collagenous structure), and four colmedins (collagen repeat plus olfactomedin containing proteins) are included in addition to prompt transmembrane collagens. (Franzke *et al.* 2003, Franzke *et al.* 2005, Snellman & Pihlajaniemi 2003.)

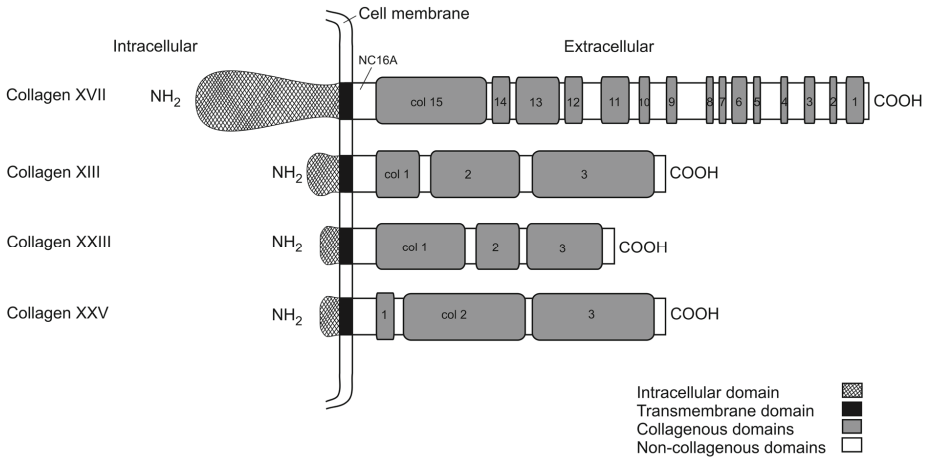

Fig. 2. Schematic presentation of the structure of transmembrane collagens. (Modified from Franzke *et al.* 2003, Franzke *et al.* 2005.)

2.3.1 Collagen XVII

Structure and synthesis

Collagen XVII was first characterized as a 180 kD bullous pemphigoid antigen having collagenous domains (Diaz *et al.* 1990, Giudice *et al.* 1991). At the protein level there is 86% homology between human and mouse collagen XVII sequences. Comparison to chick corneal collagen XVII also shows a high 78% level of homology, revealing the evolutionary importance of collagen XVII. (Li *et al.* 1993.) The collagen XVII gene is located in the long arm of human chromosome 10 and in the distal end of murine chromosome 19 (Copeland *et al.* 1993, Li *et al.* 1991). The human collagen XVII gene, COL17A1, spans about 52 kb of the genome and consists of relatively small exons, of which exons 18-56 encode the extracellular domain (Gatalica *et al.* 1997). Two different mRNA splicing variants have been detected, but their importance remains unknown (Molnar *et al.* 2000).

Collagen XVII resides in plasma membrane in type II orientation. It is composed of an intracellular part longer than in other transmembrane collagens, a short transmembrane domain and a large collagenous ectodomain. Rotary shadowing images have shown type XVII collagen as a quaver-like molecule consisting of a globular head, a central rod corresponding to the largest

collagenous domain and a flexible tail being equal to rest of the extracellular part (Hirako *et al.* 1996). The ectodomain is a flexible homotrimer that consists of 15 collagenous domains, separated by short non-collagenous domains. Respectively, in mouse collagen XVII there are 13 collagenous domains (Li *et al.* 1993). In human, the largest collagenous domain, COL15, consists of 242 amino acids, the rest varying from 15 to 45 amino acids. (Balding *et al.* 1997, Franzke *et al.* 2003, Gatalica *et al.* 1997, Giudice *et al.* 1992, Hopkinson *et al.* 1992.) The COL15 domain forms a stable triple helix. This conformation can be destabilized by glycine substitution mutations or deficiency of ascorbic acid during collagen synthesis. (Tasanen *et al.* 2000a.)

Unlike fibrillar collagens, collagen XVII triple helix formation occurs from the N- to C-terminal direction and the part of the ectodomain closest to the transmembrane, NC16A, seems to form a nucleus necessary for stable triple helix formation (Areida *et al.* 2001, Van den Bergh *et al.* 2006, Franzke *et al.* 2004). Collagen XVII is post-translationally N-glycosylated as the other collagens, and the ectodomain glycosylation site is in the C-terminus at amino acid position 1421 in NC-2 domain. Glycosylation is necessary for correct intracellular trafficking, since if glycosylation is debilitated, collagen XVII molecules accumulate intracellularly. (Franzke *et al.* 2006, Schäcke *et al.* 1998.)

Shedding

Collagen XVII exists in two triple helical forms, as a full-length 180 kD molecule and a 120 kD shed ectodomain. The shedding occurs within the NC16A domain, the amino acids 528-547 being important, but the exact cleavage site remains unclear. (Franzke *et al.* 2004, Hirako *et al.* 1998, Schäcke *et al.* 1998.) ADAMs (a disintegrin and metalloproteinase) proteinases 9, 10 and 17, also referred to as TACE (tumour necrosis factor α converting enzyme), physiologically shed the ectodomain. The TACE is the major sheddase, and the cleavage is mediated by furin by activating the ADAMs. However, lack of furin does not prevent the shedding. (Franzke *et al.* 2002, Franzke *et al.* 2004.) Regulation of the shedding occurring in plasma membrane is uncertain. However, plasma membrane microenvironment affects the shedding, as decreasing plasma membrane cholesterol levels have been shown to stimulate collagen XVII shedding (Zimina *et al.* 2005). Furthermore, post-translational phosphorylation of the extracellular serine residues perturbs binding of TACE to collagen XVII and thus inhibits the shedding (Zimina *et al.* 2007). The physiological implications of the ectodomain

shedding are not definite, but obviously it is involved in the regulation of epithelial cell motility, differentiation and detachment (Franzke *et al.* 2002, Franzke *et al.* 2003).

Ligands

Collagen XVII is a component of the hemidesmosomal adhesion complex having multiple binding partners both intra- and extracellularly. The intracellular domain of collagen XVII interacts with the β_4 integrin subunit, and FNIII (fibronectin type III) repeats of the β_4 integrin have been shown to be necessary for this interaction. (Aho & Uitto 1998, Borradori *et al.* 1997, Borradori *et al.* 1998, Schaapveld *et al.* 1998.) Plectin and BP230 are intracellular ligands of collagen XVII as well, and they both interact with the same binding site, possibly contending for it. However, BP230 and plectin cannot replace each other in hemidesmosome formation. (Koster *et al.* 2003). In cell-cell contacts the cytoplasmic domain of collagen XVII may interact with members of the actinin family, such as actinin-4 (Gonzalez *et al.* 2001). In addition to intracellular interaction with β_4 integrin, the extracellular NC16A domain binds to the α_6 integrin subunit (Hopkinson *et al.* 1995). Most recently, laminin 5 has been shown to be another extracellular ligand of collagen XVII binding to the C-terminus (Tasanen *et al.* 2004).

Localization in tissues and functional aspects

Typically, collagen XVII has been thought to be expressed in cell membranes of stratified squamous epithelia especially in the skin (Nishizawa *et al.* 1993). The expression level is not stable and can be influenced by cellular conditions such as calcium concentration (Schmidt *et al.* 2006). However, the tissue distribution of collagen XVII has been shown to be relatively wide in both specialized and simple epithelia. Besides the skin, it is expressed in ocular cornea and conjunctiva, buccal mucosa, upper oesophagus, placenta, umbilical cord, urine bladder and bronchial epithelia (Fairley *et al.* 1995, Michelson *et al.* 2000). Most recently, collagen XVII has also been shown to be expressed in the neurons of the central nervous system and in the retina (Claudepierre *et al.* 2005, Seppänen *et al.* 2006, Seppänen *et al.* 2007). Atypically, in neurons of the central nervous system, collagen XVII expression has been shown to be cytoplasmic (Seppänen *et al.* 2007). The shed ectodomain is also present in human amniotic fluid and amniotic membranes (Oyama *et al.* 2003, Schumann *et al.* 2000). At the mRNA level there

is also collagen XVII expression in salivary and thyroid glands, colon, prostate and thymus (Aho & Uitto 1999, Fairley *et al.* 1995). Collagen XVII expression is increased in epithelial malignancies, and the expression level varies within the neoplastic tissue in ameloblastomas, squamous cell carcinoma and basal cell carcinoma (Parikka *et al.* 2001, Parikka *et al.* 2003, Yamada *et al.* 1996).

Transmembrane collagen XVII is a critical component of the hemidesmosomes, providing the adherence of the epidermis to the basement membrane. It is the only molecule that directly connects the keratinocytes to the lamina densa (Masunaga 2006). Disturbance in these interactions causes dermo-epidermal tissue separation and blistering. In addition, collagen XVII is involved in signal transduction and cell migration in both physiological and pathological conditions. The largest collagenous domain of collagen XVII, COL15, mediates the cell adhesion via $\alpha_5\beta_1$ and $\alpha_v\beta_1$ integrins and promotes the transmigration of the SCC cells (Nykvist *et al.* 2001, Parikka *et al.* 2006, Tasanen *et al.* 2000a). (Van den Bergh & Giudice 2003.)

2.3.2 Collagen XIII

Type XIII collagen, which is located on plasma membranes of the cell, has a short cytosolic N-terminus and a long collagenous ectodomain (Hägg *et al.* 1998). The three collagenous domains of the ectodomain have high thermal stability as triple helices; its shedding is mediated by furin and it occurs in the NC1-domain (Snellman *et al.* 2000a, Snellman *et al.* 2000b). Besides, it has been shown in fibrosarcoma cells that the ectodomain can also be cleaved intracellularly (Väisänen *et al.* 2006). Collagen XIII is concentrated in the focal adhesions and it has multiple binding partners in extracellular matrix proteins, i.e. fibronectin, heparin, perlecan and nidogen-2, suggesting its role in cell-matrix adhesion (Peltonen *et al.* 1999, Tu *et al.* 2002). Collagen XIII is widely distributed in normal tissues and its expression is induced in malignant transformation (Snellman & Pihlajaniemi 2003, Väisänen *et al.* 2005). In mouse models it has been shown that collagen XIII over-expression leads to abnormally high bone mass in the mice, and deletions within the collagenous domain or the N-terminus cause progressive myopathy or embryonic lethality (Kvist *et al.* 2001, Sund *et al.* 2001, Ylönen *et al.* 2005).

2.3.3 Collagens XXIII and XXV

Collagen XXIII, which was first identified as mRNA expression in rat prostate carcinoma cells, has structural similarities with collagens XIII and XXV (Banyard *et al.* 2003). Collagen XXIII mRNA is found in both mature and developing organs such as human amnion, human and murine lung and cornea, and mouse skin and tendon. On protein level the tissue distribution is not that wide as it is expressed in the lung, brain, kidney and muscles of mouse. Like the other transmembrane collagens, it occurs in full-length and shed forms. Predominantly, collagen XXIII is cleaved by furin intracellularly and this is regulated by a cholesterol-dependent mechanism. The shedding of collagen XXIII is tissue-dependent, i.e. mouse skin and kidney only contain the full-length form while the brain only contains shed ectodomain, which is processed there even further. Collagen XXIII probably interacts with the basement membrane, although it is not a component of it. The function of collagen XXIII is so far unknown, but it is supposed to play a role in the formation or maintenance of cell-cell contacts. (Koch *et al.* 2006, Veit *et al.* 2007.)

Collagen XXV, also called CLAC-P (collagen-like Alzheimer amyloid plaque component precursor) is mainly expressed in neurons in the brain, but also in the heart, testis and eye at a lower level. The ectodomain of collagen XXV consists of three collagenous domains forming triple helices and it is cleaved by furin within the NC1 domain. Both full-length and shed forms of collagen XXV bind to fibrillized amyloid β peptide by electrostatic interaction. This interaction inhibits amyloid β fibril formation and thus attests the inhibitory role of collagen XXV in β-amyloid formation in Alzheimer's disease. (Hashimoto *et al.* 2002, Osada *et al.* 2005.)

2.4 Blistering diseases related to collagen XVII

Collagen XVII is associated with both autoimmune and inherited bullous diseases. Epidermolysis bullosa (EB) is a heterogeneous group of genodermatoses characterized by blistering of the skin and other tissues. The blisters usually result from minor trauma, but spontaneous blistering occurs as well. The inheritance of these tissue fragility diseases can be either autosomally dominant or autosomally recessive. Based on the ultrastructural level of blister formation in the basement membrane zone and the clinical presentation, EB diseases are currently classified into three major types (Fig. 3, Table 1), albeit a fourth subcategory of

hemidesmosomal EB has also been proposed (Fine *et al.* 2000, Pulkkinen & Uitto 1998, Uitto & Pulkkinen 2001, Uitto & Richard 2005). In EB simplex (EBS), blistering is caused by fragility of basal layer keratinocytes due to defects in keratins 5/14 or plectin genes (Coulombe *et al.* 1991, Irvine 2005, Lane *et al.* 1992, McLean *et al.* 1996, Uitto *et al.* 1996). However, a mutation causing large deletion of the collagen XVII intracellular domain has also been shown to cause a phenotype similar to EBS (Huber *et al.* 2002). The dystrophic forms of EB are characterized by scarring caused by extensive dermal blistering. Although all subtypes of dystrophic EB (DEB) are derived from mutations in the gene encoding collagen VII (COL7A1), the major component of anchoring fibrils, the clinical spectrum of disease severity is highly variable (Christiano *et al.* 1993, Uitto & Christiano 1994, Varki *et al.* 2007). The third major EB type, junctional EB (JEB) with its subtypes, in which tissue separation occurs within the basement membrane is more closely described in the Chapter 2.4.1 (Aumailley *et al.* 2006, Bruckner-Tuderman 2002, McGrath & Mellerio 2006, Uitto & Richard 2005).

In autoimmune diseases of the pemphigoid group (bullous pemphigoid, gestational pemphigoid, linear IgA bullous dermatosis, mucous membrane pemphigoid and lichen planus pemphigoid) collagen XVII is the major autoantigen, and thus the tissue separation occurs in the dermo-epidermal junction. Pemphigoid diseases vary clinically, but histologically sub-epidermal blistering is seen in all forms. (Van den Bergh & Giudice 2003, Sitaru *et al.* 2007.)

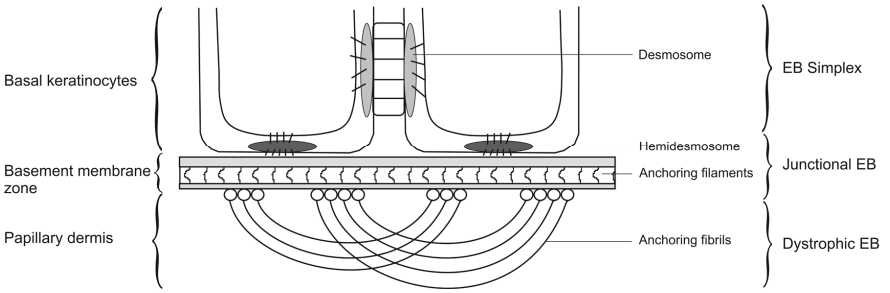

Fig. 3. Schematic representation showing the level of blister formation in major types of epidermolysis bullosa. (Modified from Varki *et al.* 2006 according to Fine *et al.* 2000, Masunaga 2006.)

Table 1. Classification of epidermolysis bullosa (EB). (Fine *et al.* 2000, Masunaga 2006, Nakano *et al.* 2002.)

EB type or subtype	Cleavage plane	Genes involved
EB simplex (EBS)		
Weber-Cockayne type of EBS	Basal keratinocytes	KRT5, KRT14
Köbner type of EBS	Basal keratinocytes	KRT5, KRT14
Dowling-Meara type of EBS	Basal keratinocytes	KRT5, KRT14
EBS with muscular dystrophy	Basal keratinocytes	PLEC1
Dystrophic EB (DEB)		
Dominant DEB	Papillary dermis	COL7A1
Recessive DEB, Hallopeau-Siemens type	Papillary dermis	COL7A1
Recessive DEB, non-Hallopeau-Siemens type	Papillary dermis	COL7A1
Junctional EB (JEB)		
Herlitz JEB	Lamina lucida	LAMB3, LAMC2, LAMA3
Non-Herlitz JEB	Lamina lucida	COL17A1, LAMB3, LAMC2, LAMA3
JEB with pyloric atresia	Lamina lucida	ITGB4, ITGA6

2.4.1 Junctional epidermolysis bullosa

Herlitz-type junctional epidermolysis bullosa

Recessively inherited Herlitz-type junctional EB (H-JEB) was previously called lethal-EB due to its severe clinical course. In the U.S. population the carrier risk for H-JEB is calculated to be one to 781, which is about half of the JEB carrier risk (Nakano *et al.* 2000). H-JEB usually leads to early death within the first or second year of life. Typically, patients have widespread erosions and blistering of the skin and mucous membranes already at birth. (Bruckner-Tuderman 2002.) Tissue fragility results from the mutations in any of the three genes encoding the polypeptides of laminin 5 (Aberdam *et al.* 1994, Mühle *et al.* 2005, Nakano *et al.* 2002, Pulkkinen *et al.* 1994, Pulkkinen *et al.* 1995, Pulkkinen *et al.* 1998). The vast majority of the mutations reported, as well as recurrent hotspot mutations, reside in the LAMB3 gene, and the LAMA3 and LAMC2 genes are equally affected. Most of the mutations in laminin 5 genes lead to premature termination codon (PTC) either by nonsense mutation or insertion/deletion resulting in frameshift. The PTC predicts the synthesis of truncated polypeptides and nonsense-mediated mRNA decay, and thus absence of functional laminin 5. (Kivirikko *et al.* 1996, Varki *et al.* 2006.) At the ultrastructural level, H-JEB

patients' skin shows markedly reduced or absent hemidesmosomes and no sub-basal dense plate (Fine *et al.* 2000).

Due to high mortality and morbidity of H-JEB patients, prenatal diagnosis and genetic counselling is a possibility for affected families (Christiano *et al.* 1997, Pfendner *et al.* 2003). However, since modifying factors can cause significant variability in the clinical course, it is suggested that the prediction of the phenotype not be based on mutation analysis alone (Mühle *et al.* 2005, Schneider *et al.* 2007). Different gene therapeutic strategies have been considered in the treatment of H-JEB patients (Bruckner-Tuderman 2002, Schneider *et al.* 2007, Uitto & Pulkkinen 2000). Recently, the prenatal LAMB3 gene delivery with two different vectors led to minor improvement in the life-span of H-JEB mice (Mühle *et al.* 2006). *Ex vivo* gene therapy has also been shown to lead to full functional correction of the JEB in a patient with mutations in the LAMB3 gene (Mavilio *et al.* 2006).

Non-Herlitz-type junctional epidermolysis bullosa

Non-Herlitz-type junctional epidermolysis bullosa (nH-JEB) is a genodermatose caused by mutations located in COL17A1 or any of the genes encoding laminin 5 polypeptides, LAMA3, LAMB3 and LAMC2. This phenotype is also known as generalized atrophic benign epidermolysis bullosa (GABEB), but according to the EB Consensus meeting this term should be withdrawn and these patients merged into nH-JEB (Fine *et al.* 2000). Since both collagen XVII and laminin 5 are crucial for hemidesmosome formation, the hemidesmosomes are rudimentary or completely lacking in nH-JEB patients' skin. Clinically, generalized blistering occurs at birth, but can improve at least partly from middle adulthood on. Blistering typically affects the extremities, but the trunk, scalp and face are usually also affected. Nail involvement as dystrophy or loss of nails is common and alopecia varies from mild to total loss of hair. Defects in enamel lead to dystrophic dentition or enamel-pitting. Recently, it has been suggested that less severe forms could be differentiated from generalized ones by collagen XVII antigen mapping (Pasmooij *et al.* 2007). (Bruckner-Tuderman 2002, Hintner & Wolff 1982.)

Most mutations in the collagen XVII gene described so far are small insertions or deletions resulting in PTC, nonsense-mediated mRNA decay and absence of collagen XVII in tissues (Bauer & Lanschuetzer 2003, McGrath *et al.* 1995, Murrell *et al.* 2007, Pasmooij *et al.* 2007, Stenson *et al.* 2003, Varki *et al.*

2006). In contrast, missense mutations or combinations of missense/nonsense mutations are more rare (McGrath *et al.* 1996, Tasanen *et al.* 2000b). Mutations in the genes encoding for laminin 5 polypeptides are missense mutations or in-frame insertions/deletions. Depending on the mutation, the phenotype varies from an EBS-like phenotype to a shortened life span. The homozygosity for PTC causing mutations tends to cause more severe disease, but the unequivocal genotype-phenotype correlation is not known so far. (Varki *et al.* 2006.) In addition, a patient with compound mutations in both COL17A1 and LAMB3 genes has been described to show more severe phenotype than patients' with disparate mutations in these genes (Floeth & Bruckner-Tuderman 1999).

Junctional epidermolysis bullosa with pyloric atresia

Junctional EB with pyloric atresia (JEB-PA) is a rare variety of JEB, characterized by congenital pyloric atresia in addition to skin blistering. The clinical severity of JEB-PA varies from post-natal lethality due to gastrointestinal complications to mild skin involvement associated with successfully operated pyloric stenosis. In addition, enamel hypoplasia, nail dystrophy and vesico-urinary tract involvements can also be seen, and associated alopecia has been described as well (Abe *et al.* 2007). Overall, the patients with JEB-PA have high mortality, but some improvements in prognosis have been made (Al-Salem 2007). (Bruckner-Tuderman 2002, Pulkkinen & Uitto 1998.)

JEB-PA is mainly caused by mutations in the ITGA6 and ITGB4 genes coding for integrin subunits α_6 and β_4 (Pulkkinen *et al.* 1997a, Pulkkinen *et al.* 1997b). Mutations in ITGB4 are more common, but there is no major clinical difference between the genes affected. Recently, mutations in plectin gene PLEC1 have also been shown to cause EB associated with pyloric atresia (Nakamura *et al.* 2005, Pfendner & Uitto 2005). Homozygous PTC mutations in the integrin genes lead to more severe phenotypes and missense mutations are possibly associated with milder cases, although their consequences are position-dependent (Nakano *et al.* 2001). Deficiency of $\alpha_6\beta_4$ integrin disturbs ligand interactions within hemidesmosomes and thus leads to depressed epidermal adhesion. (Bruckner-Tuderman 2002, Pulkkinen & Uitto 1998, Pulkkinen *et al.* 1999, Varki *et al.* 2006.)

2.4.2 The pemphigoid group of autoimmune blistering diseases

Bullous pemphigoid

Bullous pemphigoid (BP) is the most frequent autoimmune blistering disease among European population. It typically affects the elderly, men slightly more often than women, and it may have an association with other autoimmune diseases. However, no weakness in self-tolerance has been shown in BP patients (Rensing-Ehl *et al.* 2007). Clinically, BP is typically characterized by tense blisters, usually associated with severe itching. Examination of BP patient's skin by direct immunofluorescence shows linear C3 deposition along the basement membrane and in most cases IgG as well. In the case of atypical BP, the immunofluorescence stainings of salt-split skin can be used to verify the diagnosis. In BP, autoantibodies are directed to BP230 (also called as BPAG2, bullous pemphigoid antigen 2) or collagen XVII. The four major antigenic sites (referred as MCW) are clustered within the most N-terminal part of the NC16A domain of collagen XVII. The MCW-1 epitope is most often recognized by BP sera and its titre, which can be measured by enzyme-linked immunosorbent assay (ELISA), correlates with the activity of the disease. (Mihai & Sitaru 2007, Zillikens 1999.) Despite the relatively benign course of BP the morbidity is considerable. In the treatment of BP corticosteroids are used either topically in milder cases or as a systemic drug in moderate to severe disease. (Walsh *et al.* 2005, Wojnarowska *et al.* 2002.)

In the mouse model, passive transfer of IgG has been shown to induce blister formation. The autoantibody deposition alone does not induce the blistering: complement activation via classical pathway is needed. Matrix metalloproteinase-9 is necessary for blister formation, since its activation results in the release of neutrophil elastase and thus collagen XVII degradation. (Liu *et al.* 1993, Liu *et al.* 2005, Yancey 2005.) Recently, a COL17-humanized mouse model was generated, and it shows direct evidence of human BP autoantibodies causing subepidermal blistering (Nishie *et al.* 2007).

Gestational pemphigoid

Pemphigoid gestationis (PG), formerly known as herpes gestationis without any relation to herpes infection, is a rare bullous disease associated with pregnancy, with an incidence of about 1 in 50,000 pregnancies (Ambros-Rudolph *et al.* 2006).

The onset of PG varies from first trimester of pregnancy to post-partum, but most often it occurs in the second or third trimester. During subsequent pregnancies PG recurs, appears at earlier state and lasts longer. Clinically, urticarial papules and plaques with itching are seen, typically in the periumbilical area spreading to flexural extremities. Blisters develop in these erythemal areas within a few days or up to a month. At later stages of pregnancy the PG tends to heal spontaneously, although a flare-up may occur after delivery. Although the blistering can affect the foetus as well, there is no increased risk of foetal mortality related to PG. Instead, an association with premature delivery and small-for-date newborns has been reported (Mascaro *et al.* 1995, Shornick & Black 1992). Diagnostic immunofluorescence of perilesional PG skin shows typical complement C3 deposition along the basement membrane and in fourth to one-third of the cases there is also IgG deposition. Enzyme-linked immunosorbent assay (ELISA) of BP180 NC16A-IgG has proven to be useful in the follow-up of the disease and in differential diagnosis as well (Aoyama *et al.* 2007, Powell *et al.* 2005). Systemic corticosteroids are the mainstay of PG treatment, although milder cases can be effectively treated with potent topical corticosteroids and antipruritic drugs (Castro *et al.* 2006, Lin *et al.* 2001). Recently, routine foetal assessment with ultrasound in PG pregnancies was suggested due to failures in foeto-placental circulation (Dolkart *et al.* 2006). (Holmes & Black 1983, Jenkins *et al.* 1999, Lin *et al.* 2001, Shimanovich *et al.* 2002.)

As in BP, autoantibodies are directed in PG against collagen XVII and in some cases also against BP230, but without pathological relevance. PG autoantibodies mainly bind to the two epitopes of the NC16A domain, but reactivity against intracellular epitopes is also observed already at the onset of the disease. (Di Zenzo *et al.* 2007, Giudice *et al.* 1993, Herrero-Gonzalez *et al.* 2006, Matsumura *et al.* 1996, Powell *et al.* 2005.) PG is associated with some maternal and paternal HLA-types. Abnormal expression of these MHC II (major histocompatibility complex II) molecules in the placenta is suggested to have an important role in the production of autoantibodies in PG. Collagen XVII antigen is either presented by abnormal MHC molecules or in the context of paternal MHC, thus evoking an allogenic reaction (Kelly *et al.* 1989a, Kelly *et al.* 1989b). (Lin *et al.* 2001, Shimanovich *et al.* 2002.)

Linear IgA bullous dermatosis

Linear IgA bullous dermatosis (LAD) is a rare subepidermal blistering disorder affecting both adults and children. In general, there is variance in the cutaneous manifestations of LAD, ranging from papules to vesicobullous eruptions, and the symptoms of LAD can mimic other bullous diseases. The trunk is most commonly affected and the majority of patients also have mucosal involvements. The clinical picture of the childhood variant, which is referred to as chronic bullous disease of childhood, differs slightly from the adult one. In immunofluorescence LAD is characterized by linear IgA deposits along the basement membrane zone. Although LAD is usually idiopathic, drug-induced cases with similar immunopathological features have been described (Onodera *et al.* 2005). Despite its potential side effects, dapsone is the drug of choice in both adults and children in the treatment of LAD. (Guide & Marinkovich 2001, Zillikens 1999.)

The antigens targeted in LAD are heterogenous and have so far not been completely characterized. Collagen XVII, LAD285 and BP230 are the most common antigens, but there are also seven minor antigens frequently detected in LAD. Both IgA and IgG autoantibodies occur, and IgA antibodies can bind multiple antigens especially in adults, whereas the IgG response is restricted. Collagen XVII is the predominant antigen targeted by both isotypes of autoantibodies, and the epitopes are mostly located in the NC16A domain, although COL15 has also been shown to be an antigenic epitope (Schumann *et al.* 2000, Zillikens *et al.* 1999b, Zone *et al.* 1998). Both in LAD and in chronic bullous disease of childhood the IgA autoantibodies target the collagen XVII ectodomain more effectively than the full-length collagen XVII (Schumann *et al.* 2000). (Allen & Wojnarowska 2003, Zillikens 1999.)

Mucous membrane pemphigoid

In contrast to BP, PG and LAD, mucous membrane pemphigoid, previously referred to as cicatricial pemphigoid, predominantly affects mucous membranes. Most commonly it involves the ocular and oral mucosa, having an effect on the skin in only a minority of the patients. Typically, mucous membrane pemphigoid is a disease of middle-aged women, causing easily infective erosions that heal with scarring and accordingly cause strictures. (Chiou *et al.* 2007, Kolanko *et al.* 2004.) In direct immunofluorescence there are linear IgA, IgG or C3 deposits

along the basement membrane zone. Autoantibodies in mucous membrane pemphigoid are directed against collagen XVII, laminin 5, collagen VII and β_4-subunit of the $\alpha_6\beta_4$ integrin. In collagen XVII most of the mucous membrane pemphigoid autoantibodies react either within the NC16A domain or the C-terminal region (Balding *et al.* 1996, Bedane *et al.* 1997). (Mihai & Sitaru 2007, Zillikens 1999.)

Lichen planus pemphigoid

Lichen planus pemphigoid is a rare bullous skin disease, which can in some cases also involve oral mucous membranes (Solomon *et al.* 2007). In lichen planus pemphigoid bullous lesions usually appear concomitantly with lichenoid eruptions within either affected or unaffected skin. Direct immunofluorescence demonstrates linear IgG and/or complement C3 staining in perilesional skin. Antigen characterization has not unequivocally proven antigens in lichen planus pemphigoid. However, lichen planus pemphigoid sera have been shown to react with both BP230 and collagen XVII. Within collagen XVII, epitope MCW-4 in the C-terminal part of NC16A has been shown to be a unique for lichen planus pemphigoid (Zillikens *et al.* 1999a). (Zillikens 1999.)

2.5 Placental development

The placenta is crucial for normal development and survival of the foetus. Placental structures provide the physiological exchange of gases, nutrition and waste products between the foetal and maternal circulations. Besides, it produces hormones maintaining the pregnancy, i.e. human chorionic gonadotrophin and progesterone, and participates in immune protection of the foetus by redirecting maternal endocrine and immune systems. Failures in the implantation or placentation are common, and one third of conceptions end in spontaneous abortion. Correspondingly, one in five clinical pregnancies terminates in miscarriage. Later on, placental defects may also result in pregnancy threatening diseases such as pre-eclampsia. (Cross *et al.* 1994, Maconochie *et al.* 2007.) Although there are similarities in placental development between the species, remarkable differences exist as well, and there is no ideal animal model for human placentation (Carter 2007, Cross *et al.* 2003). Thus, principles of human placentation are described exclusively in following.

Human placental formation begins already at the blastocyst stage, when the cells of morula form a blastocyst in which the cell mass obtains the cavity and is differentiated into the trophoblast shell and the inner cell mass, which develops into the future embryo. As soon as the blastocyst implants to uterine endometrium on day six after conception the trophoblast proliferates to form a double-layered trophoblast. The inner layer remaining discrete is called the cytotrophoblast and the outer layer formed by fused cytotrophoblasts is the syncytiotrophoblast (Fig. 4). The villus formation begins when syncytiotrophoblast broadens finger-like to the endometrium. When cytotrophoblasts invade within and form a cytotrophoblastic shell towards the endometrium the primary villi are formed. In the early placenta, villi are composed of fibrovascular stroma and covered by the inner cytotrophoblastic layer and the outer syncytiotrophoblast. During the pregnancy the stem villi decrease and terminal villi become the majority. (Huppertz *et al.* 2003.)

The placental invasiveness in normal pregnancy is temporally and spatially regulated, since it is restricted both to the first trimester of the pregnancy and to the uterus. At the implantation site, there is a local uterine immune response to the placenta, which allows the access to maternal “supplies” but restrains over-invasion. (Hu *et al.* 2006, Moffett & Loke 2006.) The sub-population of cytotrophoblasts, intermediate (extravillous) trophoblasts, has a major role in invasion to the endometrium. In addition, they have an ability to modify decidual vessels to low-resistance vascular system. Matrix metalloproteinases have been shown to play a key role in the invasive potential of the intermediate trophoblasts (Cohen *et al.* 2006, Hurskainen *et al.* 1996). Changing of integrin expression from $\alpha_6\beta_4$ of cytotrophoblasts to $\alpha_5\beta_1$ or $\alpha_1\beta_1$ of intermediate trophoblasts has a significant effect on trophoblast invasion (Damsky *et al.* 1994, Zeng *et al.* 2007). Besides, the invasion of the intermediate trophoblasts is controlled by hormones, cytokines, transcription factors, maternal immune system as well as glucose concentration (Belkacemi *et al.* 2005, van den Brûle *et al.* 2005, Jurisicova *et al.* 2005, Renaud *et al.* 2005). (van den Brûle *et al.* 2005.)

Differently from the placenta, human amniotic membrane predominantly originates from the inner cell mass. It is composed of a single layer of epithelial cells, a thick basement membrane and an avascular mesenchyme. The trophoblasts of the chorion leave are adjacent to the amniotic membrane, and together these membranes form a chorio-amnion, more often designated as the foetal membranes. (Aplin *et al.* 1985, Huppertz *et al.* 2003, Toda *et al.* 2007.) Within the foetal membranes the foetus is surrounded by amniotic fluid. Both the

composition and origin of the amniotic fluid change during the pregnancy from transudation of isotonic foetal plasma to hypotonic fluid originating from foetal micturition (Fauza 2004). Different cell types can be detected in the amniotic fluid, and both mesenchymal and scarcer embryonic-like stem cells have been shown to be of foetal origin (Fauza 2004, Virtanen *et al.* 1981). However, most proteins in the amniotic fluid are of maternal origin (Drohse *et al.* 1998).

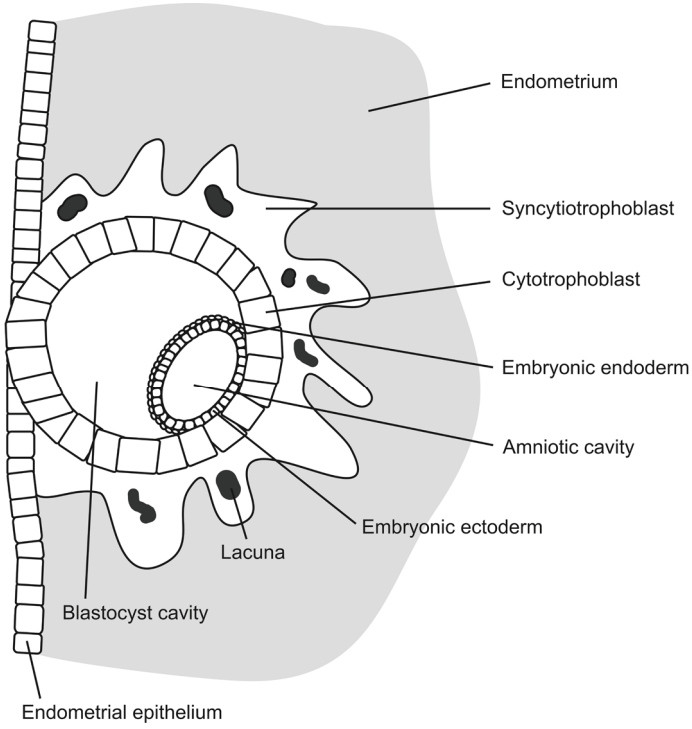

Fig. 4. Placental formation and an embryo during the first days of pregnancy. (Modified from Burkitt *et al.* 1993.)

3 Outlines of the present study

Transmembrane collagen XVII is well characterized, and its necessity for skin stability is distinctly established, as it is associated with both autoimmune and inherited blistering skin diseases. In the collagen XVII gene, missense mutations are a rarity and their molecular consequences are thus far deficiently understood. Despite the wide data about collagen XVII, the studies mainly focus on the skin, while less is known about collagen XVII in extracutaneous tissues. The aim of this work was to explore and widen the knowledge of the molecular role of collagen XVII in the pathogenesis of both autoimmune and inherited bullous diseases. The specific aims of the study were:

1. to detect novel mutations and understand the molecular mechanisms of COL17A1 missense mutations in junctional epidermolysis bullosa,
2. to study the expression pattern and molecular forms of collagen XVII in the placenta in order to understand better its role in gestational pemphigoid, and
3. to investigate the expression of collagen XVII in extracutaneous tissues, i.e. foetal membranes.

4 Materials and methods

4.1 Generation of mutated collagen XVII constructs (I, III)

Full-length cDNA for human collagen XVII (GenBank accession number M911669) was generated as described earlier (Borradori *et al.* 1997) and cloned into the NotI site of pcDNA3 (Invitrogen Corp., San Diego, CA). The glycine substitution mutations G612R, G627V (McGrath *et al.* 1996) and G633D (Tasanen *et al.* 2000b) and the deletion of the amino acids 779-787 (Chavanas *et al.* 1997) were accomplished by using the QuikChange Site-Directed Mutagenesis kit (Stratagene Europe, Amsterdam, the Netherlands) and the sense and antisense primers containing the desired mutations (Table 2). The G609D and the control substitution mutation V703M (Schumann *et al.* 1997) were generated by using the GeneTailor Site-Directed Mutagenesis System (Invitrogen Europe, Leek, the Netherlands) and the overlapping primers shown in Table 2. The generation of the desired point mutations and the deletion were confirmed by DNA sequencing.

Table 2. Primers used for site-directed mutagenesis.

Mutation	Forward primer[1]	Reverse primer[1]
G609D	5'- GGACCAAAGGGTCAAAAAGACAGCGTG GGA-3'	5'-TTTTTGACCCTTTGGTCCTTGTGGACCTG G-3'
G612R	5'-GGTCAAAAAGGCAGCGTGAGAGATCCTG GCATGG-3'	5'-CCATGCCAGGATCTCTCACGCTGCCTTT TTGACC-3'
G627V	5'-GCCAGAGAGGGCGAGAAGTCCCCATGG GACCTCG-3'	5'-CGAGGTCCCATGGGGACTTCTCGCCCTC TCTGGC-3'
G633D	5'-CCCATGGGACCTCGTGATGAGGCAGGG CCTCCTGG-3'	5'-CCAGGAGGCCCTGCCTCATCACGAGGTC CCATGGG-3'
del779-787	5'-GGACCTTCTGGAGACCCAGGAAAGCC A↓GGACTTCCCGGTACCCCTGGCCGACCA-3'	5'-TGGTCGGCCAGGGGTACCGGGAAGTC C↓TGGCTTTCCTGGGTCTCCAGAAGGTCC-3'
V703M	5'-GTCAAAGGTGACAAAGGACCAATGGGA CCACCAGGA-3'	5'-TGGTCCTTTGTCACCTTTGACACCAGGAA G-3'

[1]Mutated base underlined, deletion marked as ↓

4.2 Cell culture procedures

4.2.1 Culturing of cells (I, II, III)

COS-7 cells (African green monkey kidney cells from ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and HTR-8/SVneo cells (immortalized human trophoblast cell line (Graham *et al.* 1993)) in RPMI 1640 both containing 10% heat inactivated foetal calf serum (FCS) and 1% penicillin-streptomycin (all from Gibco, Paisley, UK). Human amniotic fluid cells originally collected for karyotype analysis at 17th week of gestation were cultured in Amnion Max (Gibco). HaCaT cells (human keratinocytes, a generous gift from Dr. N. Fusenig, German Cancer Research Center, Heidelberg, Germany) were cultured in serum-free keratinocyte medium supplemented with recombinant epidermal growth factor and bovine pituitary extract (Gibco). Cells were grown in humidified atmosphere at 37°C with 5% CO_2. Culture media were replaced every 2-3 days and the subcultures were obtained by trypsin/EDTA treatments. During the experiments the cells were cultured in the presence of fresh ascorbic acid 50 μg/ml (Fluka Chemie, Buchs, Switzerland) added for every 24 h.

4.2.2 Transfections (I, III)

COS-7 cells were grown to 90% confluency prior to transfection. Transfections were performed either by ProFection DEAE/Dextran Mammalian Transfection System (Promega, Madison, WI) or with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Respectively, 10 μg and 16 μg of DNA were used for 10 cm culture dish. The same transfection method was used in parallel experiments. For analysis, cell extracts and media were collected 48 h after the transfections.

4.2.3 Cell migration assays (II)

Transmigration assays were performed using Transwell 6.5 mm filters with 8 μl pore size (Corning Incorporated, Acton, MA). Prior the experiments the filters were equilibrated for 1h at 37°C with RPMI-1% FCS (serum reduced media). 2.5 x 10^5 HTR-8/SVneo cells were plated in 100 μl of serum-reduced media to the upper well of the Transwell system and 600 μl of the same medium was used in the lower chamber. Denatured (20 minutes at 56°C) or native recombinant COL15 (Tasanen *et al.* 2000a) was added to either the upper or lower chamber or

both. The cells were allowed to migrate for 48 h in the presence of 60 μg/ml COL15. The upper side of the filter was swabbed to remove the non-migrated cells. The cells at the bottom of the filter were fixed with ice-cold methanol and stained with 0.1% Toluidine blue. The cells were counted on three separate fields with a 10x objective.

4.2.4 Cell surface biotinylation (III)

Collagen XVII transfected cells were washed three times with PBS. The cells were surface-biotinylated with D-biotinoyl-ε-aminocaproic acid N-hydroxysuccinimide ester (Boeringer Mannheim, Mannheim, Germany) according to the manufacturer's recommendations for five minutes and then again washed carefully with PBS. After biotinylation the cells were cultured in fresh medium supplemented with ascorbic acid. The cell extracts and media were regularly collected and processed accordingly (Schäcke *et al.* 1998). The precipitates were immunostained with streptavidine coupled alkaline phosphatase (Roche) to detect biotinylated collagen XVII. (Franzke *et al.* 2002, Franzke *et al.* 2006.)

4.2.5 Immunofluorescence of cultured cells (II, III)

Cultured cells were fixed to glass coverslips for 15 minutes with pre-cooled methanol (-20°C) and air-dried. After two washes with 0.01 M PBS the cells were blocked for 30 minutes at RT with 10% FCS in PBS-glycine to reduce unspecific staining. Primary antibodies were diluted in 5% FCS-PBS-glycine as follows: vimentin 1:200, AE1/AE3 for cytokeratins 1:100 (both from DakoCytomation, Glostrup, Denmark) and polyclonal collagen XVII NC16A 1:300 (Schumann *et al.* 2000). The cells were incubated with primary antibody for 2 h at RT and then washed three times with PBS-glycine. Secondary antibodies Alexa Fluor 488-anti-rabbit (1:200) and Texas Red anti-mouse (1:300) (both from Molecular Probes, Leiden, the Netherlands) were diluted in 5% FCS-PBS-glycine and incubated for 30 minutes in the dark at RT. After washes with PBS-glycine as earlier and two rinses with distilled water, the cover slips were mounted with Immu-Mount (Thermo Electron Corporation, Pittsburgh, PA) mounting medium. The images were obtained with Zeiss Axioplan 2 Imaging Systems.

4.2.6 Inductions (II)

To study the collagen XVII expression in HTR-8/SVneo cells, the cells were treated with TGF-β_1, TNF-α (both 20 ng/ml, R&D Systems, Abingdon, UK) and PMA (phorbol 12-myristate 12-acetate) (20 ng/ml, Sigma, Saint Louis, MS). The cells were incubated for 24 h and the total RNA was isolated using the QIAamp RNA Blood Mini Kit (Qiagen, Crawley, UK). The experiments were repeated eight times. The mRNA levels of the induced cells were measured by quantitative reverse transcription-PCR analysis using collagen XVII-specific primers (Table 3) as described by Majalahti-Palviainen and co-workers (2000). Measurement of 18S RNA was used as a control.

Table 3. Primers used in quantitative real time-PCR.

Collagen XVII	18S
5'-CAAAGGGTCAAAAAGGCAGC-3'	5'-TGGTTGCAAAGCTGAAACTTAAAG-3'
5'-CCCTCTCTGGCCCATGG-3'	5'-AGTCAAATTAAGCCGCAGGC-3'
5'-FAM-TGGGAGATCCTGGCATGGAAGGC-TAMRA-3'	5'-VIC-CCTGGTGGTGCCCTTCCGTCA-TAMRA-3'

4.3 Protein extractions and immunoprecipitation (I, II, III)

Cultured cells and media were processed separately for collagen XVII protein analysis. The full-length collagen XVII was extracted from the cells as described earlier (Schäcke *et al.* 1998). Briefly, after washes the cell layer was incubated on ice with extraction buffer containing both detergent (1% Nonidet P-40) and protease inhibitors (7 μg/ml antipain, 7 μg/ml leupeptin, 14 μg/ml pepstatin, 14 μg/ml chymostatin (all from Sigma, Steinheim, Germany), 1 mM Pefablock (Merck, Darmstadt, Germany) and 10 μl/ml N-ethylmaleimide (NEM) (FlukaChemie)). The lysate was scraped from the culture dish and centrifuged to remove the cellular debris. Only the supernatant was used in analyses.

For analysing collagen XVII in the media and the amniotic fluid, they were either immediately immunoprecipitated or stored frozen with appropriate amounts of the above inhibitors. For the immunoprecipitation of the shed collagen ectodomain, the media were collected, immediately cooled, centrifuged to remove any cellular debris and supplemented with 1 mM Pefablock and 1 mM NEM. Preparatory to immunoprecipitation, 0.2 g of Protein A Sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden) was prepared according to manufacturer's

instructions, then washed once with PBS, once with PBS-0.5% Tween and once with PBS. Polyclonal NC16A antibody (Schumann *et al.* 2000) was added to properly washed Protein A Sepharose in 0.5 ml of PBS and rotated for 4 h at 4°C. The Protein A Sepharose/antibody complex was washed as earlier and 30 ml of the media were added and then rotated overnight at 4°C. After three washes as above the pellet was packed into a small column using 50 mM Tris-HCl, pH 7.0, at RT, as a column buffer. After extensive washes with the same buffer, the collagen XVII ectodomain was slowly eluted with 6 ml of 0.1 M glycine buffer, pH 3.0, at RT. The pH of the eluted fractions was immediately neutralized with 1 M Tris-HCl, pH 9.0, at RT. The human amniotic fluid was immunoprecipitated similarly, except that only 10 ml of the amniotic fluid was used.

4.4 Enzyme digestions (I, II, III)

To test the triple helical conformation of recombinant proteins and the collagen XVII from amniotic fluid with trypsin as a probe the immunoprecipitated ectodomains were incubated at incremental temperatures between 4 to 50°C using 1 or 2 degree of Celsius steps for 3 minutes. The samples were cooled to RT and treated with 10 μg/ml trypsin (Sigma) for 2 minutes and the reactions were then stopped by adding 10 μg/ml soy-bean trypsin inhibitor (Sigma). The scanning and quantitation of immunoblotted digestion products was performed by Quantity One software (BioRad, Hercules, CA).

Collagenase digestion of immunoprecipitated amniotic fluid was performed according to manufacturer's instructions with 3.8 U/μl of highly purified bacterial collagenase (Wak-Chemie Medical GmbH, Steinbach, Germany) for 2 h at 37°C. To study the deglycosylation of the recombinant mutated collagen XVII proteins, the cell extracts and the media were treated with 2% β-mercaptoethanol for 10 minutes at 60°C prior to digestion with 5000 U/ml N-glycosidase F or 2.5 U/ml endoglycosidase H (both from New England Biolabs, Ipswich, MA) overnight at 37°C.

4.5 Immunoblotting (I, II, III)

All protein samples (cell extracts and media, immunoprecipitated cell media and amniotic fluid, and fresh amniotic fluid) were ethanol precipitated, separated in 12% or 4-15 % gradient (BioRad) SDS-PAGE and transferred to the nitrocellulose membrane. For the alkaline phosphatase detection method,

unspecific binding was blocked for 30 minutes with 2% milk in Tris-buffered saline (TBS). The primary antibodies polyclonal NC16A (1:1000), Ecto-5 (1:500) (Schumann *et al.* 2000) or streptavidine coupled alkaline phosphatase (1:5000) (Roche) were diluted in 2% milk-TBS and incubated with the samples shaking overnight. After washes, secondary antibody anti-rabbit IgG (1:10000) (Sigma) was added for 2 h when detecting collagen XVII. Then, the membrane was washed again and the collagen XVII signal was detected by using NBT (nitro blue tetrazolium III crystal) (Sigma) – BCIP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt) (FlukaChemie) substrate.

For enhanced chemiluminescence (ECL) Western blotting the samples were treated as above. Non-specific binding was blocked by incubation in 10% milk-TBS for 60 minutes. After washes the membrane was incubated overnight with monoclonal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:3000) (SantaCruz Biotechnology, Heidelberg, Germany), followed by washes and incubation with peroxidase conjucated anti-mouse IgG (1:10000) (Sigma) secondary antibody. The protein signals were detected using ECL Western Blotting Detection Reagents and ECL-Hyperfilm (both from Amersham Biosciences, Little Chalfont, UK).

4.6 In situ hybridization and immunohistochemistry (II)

For the preparation of a digoxigenin-labelled cRNA probe, a cDNA fragment, Ecto-4, covering 389 nucleotides of collagen XVII (Parikka *et al.* 2003), was used. 3-5 µm thick paraffin sections were deparaffinized and pretreated with glycine and Triton X-100. After treatment with Proteinase-K (20µg/ml) (Roche) the sections were post-fixed with paraformaldehyde and acetylated in triethanolamine-acetic anhydride. Digoxigenin-labelled antisense and sense probes (500 ng/ml in hybridization buffer) were denaturated and applied to the sections (overnight, 58ºC). Post-hybridization washes were done in 2xSSC/50% formaldehyde for 30 minutes at 58˚C, 10 µg/ml RNAse A/TNE (10 mM Tris-HCl pH 7.5, 0.5 M NaCl, 1 mM EDTA) for 30 minutes at 37˚C, TNE for 10 minutes at 37˚C and 2xSSC for 15 minutes at 50˚C. The sections were equilibrated in 100 mM Tris-HCl, 150 mM NaCl (buffer I) and non-specific binding of anti-digoxigenin was prevented using normal calf serum. The hybridized probe was traced by alkaline-phosphatase conjucated anti-digoxigenin Fab-fragment (1:200) (Roche Applied Sciences, Mannheim, Germany). After washes in buffer I, the antibody was detected using Fast Red substrate (Roche Applied Sciences).

Sections were counterstained with Mayer's haematoxylin (Histolab Products, Göteborg, Sweden) and mounted with Immu-Mount mounting medium (Thermo Electron Corp.). A corresponding sense cRNA riboprobe was used as a negative control to determine specific hybridization. Images were obtained using Leica DC150 linked to Canon Powershot S50 system.

Monoclonal collagen XVII antibody NC16a-1 (diluted 1:5) was used for immunohistochemical stainings. Immunoperoxidase staining was performed on 5-10 μm thick paraffin sections. Deparaffinized sections were pre-treated with 10 mM Tris- 1 mM EDTA pH 9 buffer to retrieve antigen. Endogenous peroxidase activity was prevented by incubation for 5 minutes in H_2O_2. After a wash with 0.1% Tween-PBS the diluted antibody was applied on the sections and incubated overnight at 4˚C. Pre- or non-immune mouse serum was used as a negative control. The detection was performed according to DAKOReal EnVision Detection System (DakoCytomation): secondary antibody for 30 minutes at RT and DAB (diaminobenzidine tetrahydrochloride) -detection for 5 minutes at RT. After a rinse with distilled water the sections were counterstained with haematoxylin, dehydrated and mounted with Immu-Mount mounting (Thermo Electron Corp.) medium.

4.7 Immunoelectron microscopy (II, III)

Fresh amniotic membrane was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer with 2.5% sucrose, pH 7.4 for 2 hours. Respectively, transfected COS-7 cells were fixed in 4% paraformaldehyde in 0.1 M PBS at RT, scraped off from the substrate and further fixed for an hour. After pelleting the cells were immersed in 12% gelatine in PBS. Small tissue pieces/cell pellets were immersed in 2.3 M sucrose, frozen in liquid nitrogen and cut into thin 70-80 nm cryosections. For the immunolabelling, the sections were first incubated in 0.05 M glycine in PBS followed by incubation in 5% BSA with 0.1% gelatin (Aurion, Wageningen, the Netherlands) in PBS. Antibodies and gold conjugate were diluted in 0.1% BSA-C (Aurion) in PBS. All washings were performed in 0.1% BSA-C in PBS. Sections were then incubated with monoclonal antibody NC16a-1 (diluted 1:10) to collagen XVII for 60 minutes, followed by protein A-gold complex for 30 minutes. The controls were prepared by carrying out the labelling procedure without primary antibody. The sections were embedded in methylcellulose and examined in a Philips CM100 transmission electron microscope (FEI company, Eindhoven, the Netherlands). Images were captured by CCD camera equipped

with TCL-EM-Menu version 3 from Tietz Video and Image Processing Systems GmbH (Gaunting, Germany).

Table 4. Summary for primary antibodies used in this work.

Antibody name	Type	Dilutions	Used in
NC16A	Polyclonal	1:1000 (WB), 1:300 (IF)	I, II, III
Ecto-5	Polyclonal	1:500 (WB)	II
NC16a-1	Monoclonal	1:10 (IEM), 1:5 (IHC)	II, III
GAPDH	Monoclonal	1:3000 (WB)	II
Vimentin	Monoclonal	1:200 (IF)	II
AE1/AE3	Monoclonal	1:300 (IF)	II

Western blotting (WB), Immunofluorescence (IF), Immunoelectron microscopy (IEM), Immunohistochemistry (IHC)

4.8 Tissue samples (I, II)

All human tissue samples were collected after written informed consent. EB patients in whom the collagen XVII mutations were detected are summarized in Table 5. Early placental samples were collected from legal abortions performed for socio-economic reasons at the 9-10th week of gestation. Term placentas and amniotic membrane samples were obtained immediately after delivery from mothers with uneventful pregnancy. Fresh amniotic fluid samples and the amniotic fluid cells for culturing were collected from patients who underwent amniocentesis for foetal karyotyping or to confirm foetal lung maturity.

The Ethical Committees of the University of Freiburg (I) and Northern Ostrobothnia Hospital District (II) approved the study protocols, and the studies were carried out in accordance with the provisions of the Helsinki Declaration.

Table 5. Collagen XVII gene mutations of this study leading to glycine substitutions or in-frame deletions in the COL15 domain.

Patient	Age/Sex	Mutation	Consequences	Skin blistering	Scalp skin/hair
1	40/M	1826G>A/3781C>T	G609D/PTC	Mild acral	Normal
2	2/M	1834G>A/1834G>A	G612R/G612R	Mild generalized	NA
3	8/F	1834G>A/3800dupC	G612R/PTC	Mild generalized	Partial alopecia
4	24/M	1834G>A/3800dupC	G612R/PTC	Mild generalized	Partial alopecia
5	4/F	2336-2A>G/2336-2A>G	Inf.del/Inf.del	Mild acral	Normal
6[a]	50/F	3514ins25/1985G>A	PTC/G627V	Moderate generalized	Partial alopecia
7[b]	13/M	538C>A/2003G>A	PTC/G633D	Mild acral/generalized	Normal
8[c]	28/F	2336-2A>G/2336-2A>G	Inf.del/Inf.del	Mild generalized	Total alopecia

Premature termination codon (PTC), In-frame deletion of amino acids 779-787 (Inf.del), not available (NA)
[a](McGrath *et al.* 1996), [b](Tasanen *et al.* 2000b), [c](Chavanas *et al.* 1997)

4.9 Statistical analysis and structural alteration assays (I, II)

The data from quantitative real-time PCR of induced cells and from cell migration analysis were subjected to one-way variance analysis (one-way ANOVA) followed by either two-way or 1-sided Dunnett's *t*-test. In both analyses, $p<0.05$ was considered significant. Statistical Products and Service Solutions (SPSS)-program (Microsoft, Redmond, WA) was used for statistical analysis of the data with the help of a biostatistician, Medical Informatics Group, University of Oulu.

The structural alterations caused by glycine substitution mutations and in-frame deletion 779-787 were analysed using secondary structure prediction computer programs pSAAM (University of Illinois, Urbana-Champaign, IL), the PredictProtein Server (Cubic Biochemistry, Columbia University, New York, NY), and the Ambivalent Structure Predictor (Young *et al.* 1999) in combination with the SEG program (Wootton & Federhen 1996).

5 Results

5.1 Thermal stability of mutated collagen XVII (I)

In this study two novel glycine substitution mutations, G609D and G612R, and a new patient with a splice site mutation resulting in a nine amino acid deletion published before (Chavanas *et al.* 1997) were found. To analyse the molecular consequences of these and the previously described collagen XVII mutations in patients with JEB, the mutations were generated to expression vector coding for full-length collagen XVII as described in Chapter 4.1. Recombinant collagen XVII proteins were subjected to limited trypsin digestions at incremental temperatures to define their thermal stability. Both the wild-type and the mutated ectodomains degraded equally to 90 kD fragments already at 4°C. However, in further digestions at higher temperatures these fragments lost their stability at different stages. The glycine substituted ectodomains G609D and G612R started to unfold at 24°C and lost their stability to 50% at 28°C. The more C-terminally situated glycine substitutions G627V and G633D started the unfolding already at 22°C and lost their stability to 50% at 24°C. Meanwhile, the melting ranges of the wild-type ectodomain, the deletion mutant 779-787 and the ectodomain with a control valine substitution V703M were highly similar. Thus, the in-frame deletion of nine amino acids does not affect the thermal stability of the collagen XVII ectodomain. In contrast, the glycine substitution mutations in the COL15 domain significantly destabilize the collagen XVII ectodomain.

Computerized secondary structure prediction analyses supported the results of thermal stability assays. Glycine substitution mutations seemed to cause interruptions of the triple helix in the COL15 domain, but the nine amino acid deletion led to compensatory changes in the NC16A and NC15 domain.

5.2 Cellular localization of mutated collagen XVII (III)

In immunofluorescence analysis of transfected COS-7 cells, both the wild-type collagen XVII and that with the amino acids 779-787 deleted localized clearly on cell membranes. Instead, glycine substituted collagen XVII proteins G609D and G627V were mainly detectable as grainy intracellular staining, and only partly in cell membranes. The cellular localization was further analysed with immunoelectron microscopy in which gold labelling showed accumulation of

glycine substituted G609D and G627V proteins in the endoplasmic reticulum. In addition, some collagen XVII labelling was also seen in cell membranes, similarly to the wild type.

5.3 Post-translational modifications of mutated collagen XVII (III)

The post-translational modifications of mutated proteins were analysed from cell extract and media of transfected cells. SDS-PAGE analysis with dense gradient gel showed that the ectodomains of glycine substituted G609D, G612R, G627V and G633D migrated more slowly than the wild type. However, the nine amino acid (779-787) deletion did not cause a molecular weight shift. Thus, it is possible that glycine substituted collagen XVII molecules are post-translationally over-hydroxylated or -glycosylated.

The kinetics of the ectodomain shedding to the cell media was analysed using surface biotinylation of transfected cells. Western blot analysis of biotinylated cell extract and media showed that wild-type ectodomain was detectable in cell media within 2 hours. However, glycine substituted ectodomains G609D and G627V were more slowly detectable in cell media, and could be seen after four hours. Nine amino acid deletion 779-787 was shed similarly to the wild type. These results suggest that glycine substitution mutations prejudice the ectodomain shedding of collagen XVII. Glycosidase digestions with N-glycosidase F showed that all glycine substituted forms as well as the deleted form (779-787) of collagen XVII were sensitive to the digestion similarly to the wild-type. All these recombinant proteins were resistant to endoglycosidase H digestions. These findings indicate that glycine substituted collagen XVII is also properly N-glycosylated and processed through the Golgi apparatus.

5.4 Expression of collagen XVII in human placenta and in placental cells (II)

To study the collagen XVII expression in human placenta, both first trimester and term placentas were examined. In early placenta, both syncytial and cytotrophoblastic cells of villous epithelium expressed collagen XVII mRNA. The microvilli covering the syncytial cells remained silent for collagen XVII mRNA, but showed distinct granular positivity in immunohistochemical stainings with monoclonal collagen XVII antibody. This suggests that collagen XVII mRNA is produced within the double-layered villous epithelium, and the collagen

XVII protein is post-translationally transported to syncytial microvilli. In invasive intermediate trophoblasts, which invade into the endometrium, a strong collagen XVII mRNA signal and protein staining for collagen XVII were observed. The unorganized cytotrophoblasts of intervillous cytotrophoblastic columns also showed a clear collagen mRNA signal, but the immunostaining reaction varied from weak to strong.

In contrast to early placenta, collagen XVII mRNA expression was mainly absent in villous epithelium of term placenta. mRNA expression was only seen in syncytial knots, which consist of degenerative nuclei. Correspondingly, by immunohistochemistry collagen XVII was seen in syncytial knots. The villous epithelium showed only faint and patchy collagen XVII staining in syncytial trophoblasts, microvilli and a few cytotrophoblasts.

In addition to normal placenta, three PG placentas were studied. Morphologically they were comparable to normal placentas, except for mild villitis and some collections of immature, fibrotic villi. Equally to normal placenta, C3 complement and IgG staining were seen in villous capillaries and plasma proteins. However, collagen XVII staining in basement membrane was only observed in PG placentas. Villous epithelium showed similar mRNA expression and immunostaining signals as normal placenta.

Collagen XVII expression was also studied in HTR-8/SVneo trophoblastic cell line. RT-PCR demonstrated the presence of collagen XVII mRNA, and this was confirmed by sequencing. Quantitative real-time PCR was used to analyse the growth-factor regulation of collagen XVII in HTR-8/SVneo cells. PMA (20 ng/ml) and TGF-β_1 (20 ng/ml) induced the collagen XVII production up to 2.5-fold ($p \leq 0.05$,) whereas collagen XVII expression was not significantly affected by TNF-α (20 ng/ml).

5.5 Effect of COL15 on the placental cell migration (II)

The effect of COL15, the cell adhesion domain of collagen XVII, on the migration of HTR-8/SVneo cells was studied by using Transwell cell migration chambers. Addition of denatured recombinant COL15 to the lower chamber of the Transwell system doubled ($p < 0.005$) the transmigration of the HTR-8/SVneo cells. Instead, neither native COL15 nor the presence of COL15 in the upper or both chambers had any effect on the transmigration level.

5.6 Expression of collagen XVII in amniotic membranes (II)

Although it is known that collagen XVII is present in human amniotic membranes, its exact localization has not been shown previously (Oyama *et al.* 2003). In the *in situ* hybridization, collagen XVII mRNA synthesis was present throughout the single-layered cuboidal epithelium of the amniotic membranes. Correspondingly, the basal cellular membranes and the basement membrane zone showed intense staining for collagen XVII protein in immunohistochemical analysis in both early and term amniotic membranes. Similarly to placenta, the cytotrophoblasts expressed both collagen XVII mRNA and protein in amniotic membranes. The ultrastructural localization of collagen XVII in the hemidesmosomes of the amniotic membranes was confirmed by immunoelectron microscopy.

5.7 Collagen XVII in amniotic fluid and amniotic fluid cells (II)

It has previously been shown that collagen XVII can be found in amniotic fluid (Schumann *et al.* 2000). In this study, collagen XVII was detected by specific antibodies from unrefined amniotic fluid samples, and collagenase digestions confirmed the collagenous nature of this immunodetectable band. The triple-helical structure of amniotic collagen XVII was tested by temperature-dependent trypsin digestions, in which *in vivo* produced amniotic collagen XVII lost its stability to 50% at 44°C similarly to collagen XVII produced by cultured keratinocytes. However, the amount of collagen XVII was much higher in amniotic fluid than in the media of cultured keratinocytes. The collagen XVII amount was stable from the second to the third trimester of pregnancy since the immunoblotting signal strengths did not vary.

To analyse the production of collagen XVII in amniotic fluid cells, cells from the 17^{th} gestation week were cultured. The RT-PCR and the subsequent sequencing confirmed the presence of collagen XVII mRNA in these cells. In the Western blotting, both the full-length and shed ectodomain of collagen XVII were seen in cell extracts, whereas only the shed ectodomain was found in the cell media. In double-immunofluorescence, most of the cultured amniotic fluid cells expressed collagen XVII, which was detected both perinuclearly and faintly in cell membranes.

6 Discussion

6.1 Effects of glycine substitution mutations in COL15 on collagen XVII stability

In fibrillar collagens, glycine substitution mutations are most common, and they are located sporadically within the gene (Byers 2001). In contrast, most of the mutations known in transmembrane collagen XVII are PTC-causing. Previously, only three glycine substitution mutations in collagen XVII have been reported: G539E in the C-terminal end of NC16A, and G627V and G633D in the COL15 domain (Floeth *et al.* 1998, McGrath *et al.* 1996, Tasanen *et al.* 2000b). In this study two novel glycine substitution mutations, G609D and G612R, causing junctional epidermolysis bullosa, were described.

Since most collagen triple-helices propagate from the C- to the N-terminus, the mutations closer to the carboxyterminal end typically cause more severe phenotypical effects (Myllyharju & Kivirikko 2004). Although the triple-helix formation of collagen XVII occurs from the opposite N- to C-terminal direction, the more C-terminally situated glycine substitution mutations G627V and G633D in the COL15 domain affected the thermal stability of collagen XVII slightly more than the more N-terminally situated G609D and G612R. However, the in-frame deletion of nine amino acids did not affect the thermal stability, probably since this deletion does not hamper the correct triple helix formation.

6.2 Post-translational processing of mutated collagen XVII

It is known for other collagens that glycine substitutions can either alter the thermal stability or lead to intracellular accumulation of mutated protein. Interestingly, the only glycine substitution mutation outside the COL15 domain, G539E, prevented collagen XVII secretion in patient's keratinocytes and caused intracellular protein accumulation (Floeth *et al.* 1998). This glycine substitution mutation G539E is located in the NC16A domain within the stretch of amino acids 528-547 known to be important for sheddase recognition and cleavage, but its effect on the collagen XVII shedding has not been further investigated so far and remains unknown.

Our results show that glycine substitution mutations in the COL15 domain of collagen XVII result similarly in partial intracellular accumulation of mutated

proteins. However, the glycine substituted proteins seemed to be normally processed through the endoplasmic reticulum and Golgi apparatus, although their slightly higher molecular weight indicated some over-modification. In contrast to G539E secretion in keratinocytes, G609D, G612R G627V and G633D ectodomains were released from the COS-7 cell surface, although the shedding was slower than in the wild-type ectodomain. The processing of mutated collagen XVII with the nine amino acid deletion was not altered compared to the wild type.

The largest collagenous domain, COL15, significantly stabilizes the structure of collagen XVII (Areida *et al.* 2001, Tasanen *et al.* 2000a). Most of the glycine substitution mutations known so far are within the COL15 domain, which can be seen as quite untypical among the collagen superfamily. This could either be a fact or indicate that there might be more glycine substitution mutations in smaller collagenous domains, which do not destabilize the molecule sufficiently to cause a disease phenotype and thus remain undetected.

6.3 Collagen XVII in human placenta

Earlier studies on placentas in patients with gestational pemphigoid have claimed that collagen XVII is only expressed by extravillous cytotrophoblasts since the second trimester of pregnancy (Kelly *et al.* 1988, Ortonne *et al.* 1987). However, these studies were performed using sera from BP patients, which may explain the discrepancy between the studies. In our work, collagen XVII was for the first time shown to be expressed already at the first trimester of pregnancy. Both *in situ* hybridization and immunohistochemistry showed that collagen XVII is expressed by syncytial trophoblasts and cytotrophoblasts of the first trimester placenta. Thus, these findings could explain the earlier onset of PG in patients' subsequent pregnancies in which the symptoms of PG can occur in the first trimester when the collagen XVII is already expressed in placenta.

No increased risk for foetal mortality has been reported in PG pregnancies, but both small-for-date and low birth weight infants as well as premature deliveries are associated with PG (Mascaro *et al.* 1995, Shimanovich *et al.* 2002). The growth retardation is suggested to be caused by antibody deposition in the placental bed leading to placental insufficiency and changes in umbilical artery flows (Dolkart *et al.* 2006). The three PG placentas analysed in this work showed no difference in collagen XVII expression levels compared to normal placenta. However, mild villitis and some immature villous structures were seen under light microscopy while the placentas were otherwise morphologically normal.

Although no apparent morphological abnormalities were found, autoantibodies against collagen XVII in the placenta may lead to its dysfunction and thus explain the placental insufficiency and the minor clinical findings in some of the PG newborns. In obstetrical literature no attention is paid to the placental functions in PG pregnancies, and suggestions for frequent foetal assessments have been made only recently (Dolkart *et al.* 2006).

The largest collagenous domain COL15 of collagen XVII has been shown to promote physiological adhesion of epithelial cells and migration of malignant SCC cells (Parikka *et al.* 2006, Tasanen *et al.* 2000a). Placental cell migration and its invasiveness to the uterine endometrium can be seen as a strictly controlled physiological counterpart of malignant tumour cell invasion. By transmigration experiments using cytotrophoblastic HTR-8/SVneo cell line, it was shown that recombinant COL15 promotes the transmigration of invasive extravillous cytotrophoblasts. Denatured COL15 induced the migration of cytotrophoblasts, whereas native COL15 had no effect on migration, similarly to earlier findings in SCC cell migration studies (Parikka *et al.* 2006). Thus, these findings indicate that collagen XVII may have a role already at early stages of embryogenesis when extravillous trophoblasts invade into the maternal decidua.

6.4 Collagen XVII in amniotic membranes and amniotic fluid

The major function of the amniotic membranes is to protect the developing embryo against surrounding pressure. This is comparable to one of the functions of the skin, to protect the internal body. In addition, the amniotic membrane is structurally very similar to skin, although covered only by a single-layered epithelium (Aplin *et al.* 1985, Champliaud *et al.* 1996, Oyama *et al.* 2003). In this work, immunohistochemical and *in situ* hybridization studies showed collagen XVII expression in human amniotic membranes. This was further localized to hemidesmosomes by immunoelectron microscopy. Amniotic epithelium is rich in hemidesmosomes and their network plays a critical role in the mechanical integrity of the amniotic membranes (Aplin *et al.* 1985, Behzad *et al.* 1995). In normal pregnancies matrix biosynthesis of amniotic membranes continues until term, and the membrane rupture prior to parturition is not a result of degradation (Aplin *et al.* 1986). However, preterm rupture of foetal membranes is supposed to be due to matrix weakening (Guller *et al.* 1995). Low vitamin C levels in maternal plasma have been shown to be associated with preterm membrane ruptures, possibly by inhibiting the reactive oxygen species from activating

collagenolytic enzymes (Aplin *et al.* 1986, Woods *et al.* 2001). Since an adequate vitamin C level is a necessity for proper collagen synthesis, its deficiency could also directly affect continuing biosynthesis of collagens in amniotic membranes and thus result in their preterm disintegrity.

The shed ectodomain of collagen XVII has been shown to be a constituent of amniotic fluid (Schumann *et al.* 2000). It was shown here that *in vivo* produced collagen XVII is either very stable or produced throughout the pregnancy. Collagen XVII expression was also detectable in cultured amniotic fluid cells. This indicates that these cells may, in addition to amniotic membranes, be a source of collagen XVII in amniotic fluid. The collagen XVII amount in amniotic fluid was much higher than in the media of cultured keratinocytes. The collagen XVII amounts were also high in amniotic fluid samples prior to parturition, although collagen XVII mRNA and protein levels were weaker in term placenta than in early pregnancy. It is possible that these significant amounts of collagen XVII in the amniotic fluid are not just “leftovers”, but that collagen XVII might have an active functional role, although unknown so far.

7 Conclusions

Collagen XVII is a target molecule in both autoimmune and inherited blistering skin diseases. As an autoantigen it is involved in a rare pregnancy-associated gestational pemphigoid as well as in the more common bullous pemphigoid typically affecting the elderly. Junctional epidermolysis bullosa is more of an entity than a well-known disease even among physicians, but its molecular basis is rather well studied. Since inherited skin diseases are also considered as potential targets for gene therapy, widening knowledge about these inherited and autoimmune diseases may further help and improve in understanding the mechanisms and in developing potential therapies in other diseases as well.

In the present study, the following results were gained:

1. Two novel glycine substitution mutations causing junctional epidermolysis bullosa were described. These and two other glycine substitutions were shown to decrease the thermal stability of the collagen XVII ectodomain and to disturb the triple helical structure.
2. Glycine substitution mutations led to intracellular accumulation and affected the post-translational modifications of the synthesized collagen XVII.
3. A gestational pemphigoid autoantigen, collagen XVII, was shown to be expressed in the placenta already in the first trimester. Further, collagen XVII was shown to promote the migration of invasive intermediate trophoblasts, thus having a possible role in placental formation.
4. *In vivo*-produced collagen XVII ectodomain in amniotic fluid was found to be structurally stable and possibly produced in amniotic fluid cells and/or amniotic membranes. In addition, the ultrastructural localization of collagen XVII in amniotic membranes was specified to hemidesmosomes.

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