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**Laminin 332 in junctional epidermolysis and as autoantigen in mucous membrane
pemphigoid
(Part I - Chapter 9)**

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ABSTRACT

Laminin 332, with chain composition $\alpha 3\beta 3\gamma 2$, is a component of basement membranes localized beneath specialized epithelia with secretory or protective function. This protein plays a pivotal structural role as it directly connects basal epithelial cells to the underlying extracellular matrix molecular networks. Its deficiency in human inherited and autoimmune disorders has emphasized the importance of laminin 332 for the structural integrity of the dermal-epidermal junction in skin. The junctional form of epidermolysis bullosa, a skin disease characterized by loss of cohesion between the epidermis and the dermis that results in blister formation and chronic wounds, is caused by genetic mutations in several different genes encoding proteins of the epidermal adhesion complex such as the three chains of laminin 332. Besides, autoimmune disorders affecting laminin 332 belong to the mucous membrane pemphigoid heterogeneous group of rare autoimmune blistering disease. Anti-laminin 332 autoantibodies cause erosive lesions followed by scarring of the skin and mucous membranes. This chapter reviews the molecular characteristics of laminin 332, its organisation within basement membranes and its involvement in bullous diseases.

Key words: laminin 332, junctional epidermolysis bullosa, mucous membrane pemphigoid

INTRODUCTION

Laminins are large extracellular glycoproteins that are important components of all basement membranes. They are involved in several biological processes including self-polymerization, binding to the extracellular matrix (ECM) and cellular interactions [42, 7]. All laminins are composed of three different gene products, termed α , β and γ chains assembled into a cross-shaped heterotrimer $\alpha\beta\gamma$. The three chains assemble within the endoplasmic reticulum through their C-terminal domains to form a triple stranded α -helical coiled coil rod [10, 63]. Sixteen laminin isoforms of different subunit composition selected from five individual α chains ($\alpha 1$ to $\alpha 5$), three β chains ($\beta 1$ to $\beta 3$), and three γ chains ($\gamma 1$ to $\gamma 3$), are known with variable cell and tissue specific expression, and they are differentially recognized by cellular receptors [8]. All laminin α chains possess a large globule at the carboxyl-terminal end that consists of five similar domains LG1 to LG5 each containing about 200 residues [104]. Laminin 332, composed of the $\alpha 3$, $\beta 3$, and $\gamma 2$ chains, is an epithelial-basement membrane specific variant. The $\alpha 3$ chain is encoded by the LAMA3 gene, which has two transcript variants $\alpha 3A$ and $\alpha 3B$. As the truncated LAMA3A variant is expressed and incorporated into the laminin 332 heterotrimers [90], we will refer to the $\alpha 3\beta 3\gamma 2$ trimer as representing laminin 332 in this chapter. Its main role in normal tissues is the maintenance of epithelial–mesenchymal cohesion in tissues exposed to external forces including skin and stratified squamous mucosa [88, 87]. The $\alpha 3$ chain is also found in laminin 311, an isoform of composition $\alpha 3\beta 1\gamma 1$, found in association with laminin 332 in the dermal-epidermal junction [61, 18]. In skin, laminin 332 was shown to be the major component of anchoring filaments [88], where it mediates cell adhesion via interaction of the LG1-3 triplet domain with both $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins [15, 100, 84, 86].

The laminin 332 discover in the 90's drawn the scientific community's attention as evidences of this laminin isoform involvement in human heritable and autoimmune disorders were rapidly provided, demonstrating the importance of laminin 332 for the structural integrity of the dermal-epidermal junction [113, 27, 62, 36, 48]. The junctional form of epidermolysis bullosa (JEB) is a skin disease characterized by loss of cohesion between the epidermis and the dermis that results in blister formation and chronic wounds. JEB is caused by genetic mutations in several different genes encoding proteins of the epidermal adhesion complex

such as the three chains of laminin 332 (LAMA3/LAMB3/LAMC2) [47]. Besides, autoimmune disorders affecting laminin 332 belong to the mucous membrane pemphigoid (MMP) heterogeneous group of rare autoimmune blistering disease. Anti-laminin 332 autoantibodies cause erosive lesions followed by scarring of the skin and mucous membranes [50, Caux and Prost, Part III-Chapter 14]. Animal models of JEB have been developed to clarify the pathogenic mechanisms and develop therapeutic strategies. These models exhibited severe skin detachment with perinatal lethality. *Lama3* and *Lamc2* KO mouse models have been generated [89, 69]. Furthermore, spontaneous mutant dog horse and mouse models whose laminin genes are inactivated have also been described [51, 99, 70, 14]. The pathogenicity of anti-laminin 332 antibodies has also been documented in animal models. Passive transfer of anti-laminin 332 IgG to neonatal or adult mice induced subepidermal blisters of skin and mucous membranes that mimicked clinical, histological, and immunopathologic features seen in MMP patients [58, 53].

LAMININ 332 ORGANISATION IN THE DERMAL EPIDERMAL JUNCTION

In skin, laminin 332 is synthesized by keratinocytes as a high molecular weight precursor protein of 460 kDa. After secretion and deposition into the ECM, the $\alpha 3$ and $\gamma 2$ chains undergo maturation events consisting in specific processing to smaller forms [60]. The 190-200 kDa $\alpha 3$ chain ($\alpha 3_{200}$) can be successively processed at both C- and N-terminal extremities producing 165 ($\alpha 3_{165}$) and 145 kDa ($\alpha 3_{145}$) maturation products. The 155 kDa $\gamma 2$ chain ($\gamma 2_{155}$) is processed in the N-terminal region leading to a 105 kDa product ($\gamma 2_{105}$) (figure 1A). Processing of the $\alpha 3$ chain consists of cleavage of the C-terminal globular domains 4 and 5 (LG45) within the spacer between LG3 and LG4 [38, 106]. In vitro studies using human laminin 332 have revealed that enzymes involved in the processing include plasmin, MMP-2, MT1-MMP and the C-proteinase family of enzymes, especially mammalian tolloid (mTLD) and the bone morphogenic protein 1 (BMP-1) [38, 3, 112]. N-terminal amino acid sequencing of human LG45 purified from the conditioned medium of human keratinocytes revealed that the $\alpha 3$ chain is cleaved between Q1337 and D1338 within the hinge region between LG3 and LG4 suggesting that a proteolytic cleavage site of the LG45 domain matches the minimal consensus sequence LLQD [107, 25]. It is not known yet what endopeptidase catalyzes the hydrolysis of this cleavage sequence, and the existence of additional proteolytic cleavage sites has been suspected both in the hinge and the adjacent regions of the LG3 and LG4 domains [104, 106, 3, 9]. This hypothesis is reinforced by experiments showing that punctual mutations within the spacer region and/or deletion of the sequence LLQD did not protect the $\alpha 3$ LG3-LG4 linker from cleavage [3, 9]. Further cleavage occurs in the N-terminal LE region of the $\alpha 3$ chain releasing the full short arm which might be important for laminin 332 function (Fig. 1A) [18, 3]. A mutation causing an N-terminal deletion of 226 amino acids in the human $\alpha 3A$ isoform was identified in the laryngo-oncho-cutaneous syndrome, a rare autosomal recessive disorder characterized by chronic production of vascularised granulation tissue [67]. Cultured keratinocytes obtained from patients revealed that heterotrimeric laminin 332 carrying the mutant $\alpha 3$ chain is assembled and secreted. The mutated laminin was regularly expressed in the skin basement membrane and only very subtle ultrastructural changes were seen such as focal widening of the lamina lucida in places where hemidesmosome (HD) plaques were smaller. These studies suggest that the laminin $\alpha 3$ N-terminal domain may be a key regulator of the granulation tissue response during wound healing. In human skin laminin 332, the processing of the $\gamma 2_{155}$ chain leads to the $\gamma 2_{105}$ subunit and was shown to be

achieved by BMP-1 and mTLD metalloproteinase's [60, 110, 3, 92, 112]. The N-terminal processing is complex as it occurs within a disulfide-linked loop of the L4 motif followed by reshuffling of disulfide bonds for release of the cleaved fragments [92]. Laminin 332 was shown to be the major component of anchoring filaments in skin [88] where it mediates cell adhesion via interaction of the $\alpha 3$ carboxyl-terminal LG1-3 triplet domain with both $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins to form HDs [15, 100, 84, 72], while the N-terminal short arms connect to basement membrane components. Laminin 332 can be incorporated into the basement membrane through at least two mechanisms (Figure 1B). The first involves cross-linking of laminin 332 with laminin 311 ($\alpha 3\beta 1\gamma 1$) in the skin basement membrane [18]. The complex of laminin 311 with laminin 332 is most likely to derive from an interaction of domain LN in the $\beta 3$ chain of laminin 332 with domain LE of the $\alpha 3$ chain short arm in laminin 311. As seen on rotary shadowing electron micrographs of the complex, the LN domain of the short arm interacts with a laminin 311 domain near the intersection of the laminin 311 short arms [18]. These complexes are most likely stabilized by a disulfide bridge between an unpaired cysteine in domain LN of the $\beta 3$ -chain and domain LE of processed $\alpha 3$ in laminin 311 (Figure 1B). According to the 3-arm interaction hypothesis of laminin polymerization [22], the dimers could self-associate. The second mechanism reports a direct interaction between anchoring filaments and anchoring fibrils. Anchoring fibrils are disulfide bond stabilized dimers of type VII collagen [13]. Monomeric laminin 332 as well as the laminin 332/311 dimer directly bind the amino-terminal globular domain NC1 of type VII collagen and the interaction is likely to occur within the short arm of the $\beta 3$ and/or $\gamma 2$ subunit [21, 87] (Figure 2). Maturation is therefore important for the function of laminin 332 in the establishment and maintenance of the skin basement membrane structural integrity. However, recent studies have been conducted to elucidate the role of the cleavable domains in precursor laminin 332 [85]. A potential function for the tandem LG45 domains was suspected based on the ability of laminin 332 to trigger distinct cellular events depending on the level of processing of its $\alpha 3$ chain. While laminin 332 that lacks LG45 is found in mature basement membranes where it has an important function in the nucleation and maintenance of HDs through $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrin interactions [52, 37, 108], laminin 332 with intact LG45 ($\alpha 3_{200}$) was found in migratory/remodelling situations such as epidermal repair [89, 32]. Epidermal injury activates the transcription and deposition of laminin 332 into the provisional matrix by the leading keratinocytes in the process of epidermal outgrowth and migration at the wound edge [90, 52, 44]. Noteworthy, $\alpha 3_{200}$ laminin 332 is found in this provisional matrix but is absent from mature basement membranes [98, 52, 37]. Laminin 332 with an $\alpha 3_{200}$ chain is also found in the ECM of migrating keratinocytes in vitro [77, 26]. Recently, laminin 332 comprising an $\alpha 3_{200}$ chain was proposed to be involved in the invasion of squamous cell carcinomas in vivo [105]. LG45 in precursor laminin 332 was suggested to have a function in the deposition of laminin 332 [98, 105]. A study reported a missense mutation in the LAMA3 gene affecting LG4 in a patient with a mild non-Herlitz JEB phenotype [94]. This mutation resulting in G1506E triggers an imperfect local protein folding which, without impairing trimerization of the coiled coil, causes laminin 332 intracellular accumulation within the endoplasmic reticulum. Only a small amount of the laminin 332 harboring the mutated $\alpha 3$ chain is secreted and physiologically processed thus providing partial adhesion functions and explaining the mild phenotype. Therefore structural changes caused by mutations of this highly conserved residue throughout laminin LG4 domains highlights potential important functions of LG45 in laminin secretion. It was also proposed that precursor laminin 332, together with integrin $\alpha 3\beta 1$,

plays a central role in cell migration [38, 32, 79]. Syndecans have been described as cellular receptors potentially involved in these mechanisms [109, 79, 5, 101, 16]. Information concerning the precursor γ_{2155} chain is low however its involvement in keratinocyte migration was suggested [26, 75, 76].

JUNCTIONAL EPIDERMOLYSIS BULLOSA

The important role of laminin 332, and of the $\alpha 6\beta 4$ integrin, in epidermal-dermal adhesion was early confirmed by the finding that mutations affecting either of these molecules cause JEB, a genetic disorder that affects skin and mucous membranes [17]. It is characterized by a mesenchymal-epithelial separation within the lamina lucida and by hemidesmosomal abnormalities. JEB is generally divided into 3 subtypes: Herlitz (lethal), non-Herlitz (nonlethal) and JEB with pyloric atresia [4, 29, 114, 35]. All of them are inherited in an autosomal recessive manner [93] except for a single case recently reported [2]. Herlitz JEB is caused by a complete absence of laminin 332 [65, 81, 6] while non-Herlitz is caused by missense mutations leading to a reduction in functional laminin 332 or complete absence of collagen XVII [65, 81, 115, 6]. JEB with pyloric atresia is caused by a genetic mutation of $\alpha 6$ or $\beta 4$ subunits that are the main receptors for laminin 332 located beneath HDs [114, 91]. The majority of mutations identified to date in both H-JEB and nH-JEB reside in the three genes that encode the $\alpha\beta\gamma$ chains of laminin 332, LAMA3 α , LAMB3 and LAMC2 respectively [111, 74]. Normally, these three genes are expressed during all developmental stages of the skin including all steps of adhesion, proliferation and differentiation [31]. Nonsense or frameshift mutations of any of the three laminin 332 genes causing a premature termination codon (PTC) usually lead to a decrease in transcript levels due to nonsense-mediated mRNA decay [40]. If such mRNAs are translated, truncated polypeptides are produced that are incapable of participating in the formation of a functional heterotrimer. Other mutations that may occur in the laminin 332 genes include missense mutations, exon-skipping mutations and in-frame insertions or deletions. In these cases, translation of the resulting mRNA results in structurally imperfect protein production, which still allows secretion of more or less functional laminin 332 [96]. Most mutations related to the JEB are found in the LAMB3 gene and mainly in the exons encoding the N-terminal part of the $\beta 3$ chain. The LAMB3 portion coding for the C-terminal α -helical domain, which is involved in subunit assembly contains much less mutations [73, 97]. There are particularly two recurrent « hotspot » LAMB3 mutations, R635X and R42X leading to PTCs which account for the half of all LAMB3 mutations and result from a C-to-T transition [49, 73, 82]. In the LAMC2 gene most of the mutations are found within the LE domain and the L4 module and rarely in the C-terminal α -helical domain [96]. Some mutations are also found within the LG1-5 globular domains of the LAMA3 gene. Particularly, the LG3 mutation K1299X, which affects the charged amino acid lysine at position 1299 is related to a severe phenotype seen in the homozygous human patients [78, 73, 45]. As the $\alpha 3$ chain is found in laminins 311 and 321, mutations in LAMA3 gene also affect these two laminins' functions [96]. Based on the clinical severity, JEB patients with laminin 332 mutations have been classified into the Herlitz and non-Herlitz types and there are some general « rules » regarding phenotype-genotype correlations [30]. In general terms, patients harbouring PTC causing mutations on both alleles (homozygous or compound heterozygous) suffer from the severe H-JEB [81, 1]. As a result of these mutations the synthesis of one laminin 332 chain is abolished and no functional trimeric laminin 332 is produced [83, 66, 80]. The PTC mutations in H-JEB result in the absence of laminin 332

within the dermal-epidermal junction as confirmed by staining with laminin 332 specific monoclonal antibodies [39, 113, 68]. Examination of skin biopsies from H-JEB patients by electron microscopy reveals small rudimentary or absent HDs, as well as cleavages through the lamina lucida [103]. The fact that laminin 332 is involved in the regulation of motility and proliferation of keratinocytes predicts that its absence can affect the normal wound healing process causing chronic erosions and formation of granulation tissue. All resulting laminin 332 defects cause a generalized blistering and early death of the patient, usually during the first year of life. The epidermis of an affected infant can be extensively peeled away only on simple handling, while in many cases there is death by overwhelming infection, caloric deprivation or from organ failure deriving from complications of the disease [73]. nH-JEB patients often carry a PTC on one allele and a missense or in-frame splice site mutation on the alternate allele. In these cases there is often a milder phenotype due to the production of partial or abnormal laminin 332. This is in accordance with immuno-fluorescence mapping studies showing diminished expression of laminin 332 within the dermal-epidermal junction of these patients [116]. The nH-JEB phenotype is characterized by life-long blistering in a distribution that predominates in sites exposed to friction, trauma or heat, atrophic scars, hypopigmentation or hyperpigmentation at sites of healed blisters, incomplete alopecia, dystrophic nails and dental abnormalities [30, 24, 116]. The course and prognosis of nH-JEB depend on how severely affected the target protein is. Thus, some patients have a mild phenotype and normal lifespan (nH-JEB localized) and others have a severe disease and an increased risk of squamous carcinoma development and death (nH-JEB generalized) [46, 47].

MUCOUS MEMBRANE PEMPHIGOID

Mucous membrane pemphigoid (MMP) is a heterogeneous group of rare autoimmune blistering disease and is characterized by the presence of autoantibodies to various components of the dermal–epidermal anchoring complex causing erosive lesions followed by scarring of the skin and mucous membranes [50]. On the basis of the 2002 international consensus [19], MMP includes blistering diseases with preferential mucous membrane involvement. Patients with MMP may exhibit linear deposits of IgG and/or IgA autoantibodies and/or complement fragments in epithelial basement membranes. Subtypes of MMP correspond to the nature of the antigen targeted such as BP230 and BP180, laminin 332 and laminin 311, type VII collagen, or the $\alpha 6$ and $\beta 4$ integrin subunits [see Caux and Prost, Part III, Chapter 14; 50]. Early studies revealed that laminin 332 is a target antigen in about 25% of MMP patients and most patients have IgG antibodies against the α subunit of the heterotrimer [55, 48] that recognize both laminin 332 and laminin 311 [20]. The pathogenicity of anti-laminin 332 antibodies has been documented *in vitro* and *in vivo*. Passive transfer of anti-laminin 332 IgG to neonatal or adult mice induced subepidermal blisters of skin and mucous membranes that mimicked clinical, histological, and immunopathologic features seen in MMP patients [58]. Mice injection of Fab fragments directed against laminin 332 produced the same results as well as injection in mice lacking complement, mast cells or T cells, suggesting that the antibodies induced epidermal detachment in a non-inflammatory and direct manner [53, 58]. An experimental human skin graft model was also used and revealed that patients' anti-laminin 332 autoantibodies induced subepidermal blisters [54]. Assaying for anti-laminin 332 reactivity is of particular importance as 25% of patients with laminin 332-specific antibodies are suspected to develop a malignancy [59, 28]. While direct immunofluorescence microscopy of patients' epidermis and mucosal epithelium is still the

gold standard for the diagnosis of pemphigoid diseases, diagnosis can be made serologically today [95]. Patients with anti-laminin 332 antibodies display circulating IgG autoantibodies that bind to the dermal side of 1M salt-split skin by indirect immunofluorescence [59]. Western blot analysis have revealed that anti-laminin 332 autoantibodies most often react with the $\alpha 3$ chain [55, 48] and less frequently with the $\beta 3$ and/or the $\gamma 2$ chain [33, 55, 34, 102, 64]. However the exact mechanism is not known yet, it is most likely that antibodies against the $\alpha 3$ chain are disrupting the interaction with the keratinocyte integrins. Moreover, it is possible that autoantibodies against the $\beta 3$ and $\gamma 2$ chains may disrupt the interaction between laminin 332 and type VII collagen; as laminin- $\beta 3$ is thought to mediate this binding [87, 21]. Neither specific epitopes nor laminin domains targeted by anti-laminin 332 autoantibodies have been precisely identified so far and the use of recombinantly produced laminin domains will probably be helpful in such autoantibodies characterisation. Immunoprecipitation with radiolabelled human keratinocytes has been reported to be the most sensitive technique for the detection of serum autoantibodies against laminin 332, and immunoblotting using human keratinocytes' ECM or purified laminin 332 was used as a convenient method to identify the laminin subunit involved [20, 41, 56, 43]. Enzyme-linked immunosorbent (ELISA) based assays will probably soon replace these time- and reagent-consuming methods. Some studies conducted so far have reported patients' serum samples analysis in ELISA assays using either purified laminin 332 or keratinocyte ECM as a substrate [11, 56, 12]. The most recent study [12] reported the analysis of serum samples from 154 patients with MMP and 89 control individuals. The authors found that 20% of the MMP patients tested had serum anti-laminin 332 autoantibodies. Interestingly, these appeared to be present in a subset of patients with a severe form of the disease.

CONCLUSION

Dermatological pathologies related to laminin 332 are most often severe and invalidating, highlighting the important structural role of this multifunctional basement membrane protein. Intense research conducted in the last years has allowed to decipher its molecular characterisation leading to a better comprehension of the main associated diseases' physiopathology. Future studies will probably allow discovery of additional unexpected functions for this protein. For instance, a recent study has reported that the expression of laminin-332 at the dermal-epidermal junction enables a precise control of mechanosensitivity of the sensory endings that enter the epidermal layer to contact keratinocytes [23]. The laminin 332 mediated local suppression of axonal branching and mechanotransduction was suggested to prevent hypersensitivity of sensory axons entering the epidermis therefore playing a role in pain transmission. From its identification in the 1990's [88, 15, 113, 71] to nowadays, laminin 332 has stimulated incessantly increasing interest in the scientific community due to its multifunctional properties and its involvement in human physiological processes and pathologies.

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Abbreviations: ECM, extracellular matrix; JEB, junctional epidermolysis bullosa; MMP, mucous membrane pemphigoid; HD, hemidesmosome

Legends for figures

Figure 1

Molecular organisation of laminin 332 in the dermal epidermal junction. (A) Structure of human laminin 332 (modified from 85). Laminin 332 is composed of three subunits $\alpha 3A$, $\beta 3$ and $\gamma 2$. The large LG structure located at the C-terminal end of the $\alpha 3$ chain contains five repeating LG domains. The first three repeats (LG1-3) interact with $\alpha 3\beta 1$, $\alpha 6\beta 1$ and $\alpha 6\beta 4$ integrins while the last two (LG45) contain binding sites for syndecan-1 and -4. Laminin 332 is synthesized as a precursor molecule that undergoes maturation by proteolytic processings at the $\alpha 3A$ chain N- and C-terminus as well as at the $\gamma 2$ chain N-terminal extremity. The cleavage sites are indicated by arrows as well as enzymes involved identified so far. (B) Model for laminin 332 assembly at the dermal-epidermal junction. Theoretical assembly of laminins-332 and -311 in the anchoring complexes.

Figure 2

Supramolecular organisation of anchoring complexes (A) Transmission electron microscopy micrograph of human skin allowing the visualisation of the lamina lucida (LL), lamina densa (LD), hemidesmosome (HD), anchoring filaments (AF), and anchoring fibrils (af), bar 100 nm. (B) Schematic representation of anchoring complexes. Laminin 332 (pink) and laminin 311 (orange) within the anchoring filaments (AF) connect to hemidesmosomes (HD), span the lamina lucida and interact with anchoring fibrils (af) within the lamina densa (LD). Dermal anchoring fibrils are made of type VII collagen. This representation focuses only on anchoring complexes and does not show the molecular network formed with collagen IV, nidogen, laminin 511 and perlecan, which also significantly contributes to the structural properties of the dermal-epidermal junction.

Figure 2