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REVIEW ARTICLE

Expression of Laminin 332 in Vesicant Skin Injury and Wound Repair

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ABSTRACT

Sulfur Mustard (SM) is a potent vesicant or blistering agent. It is a highly reactive bi-functional alkylating agent that cross links proteins, DNA, and other cellular components. Laminin 332 is a heterotrimer glycoprotein and a crucial skin component that attaches the epidermal basal keratinocytes to the dermis. SM wounds histologically appear similar to Epidermolysis Bullosa (EB), human genetic blistering diseases that involve genetic changes in laminin 332. The specific mechanism of action of SM exposure is unknown, but there are several key similarities between vesicant induced cutaneous injury and the Junctional form of EB (JEB) cutaneous injury: 1) Initial alkylation causes blistering similar to JEB; 2) Initial injury is followed by protease activation and prolonged inflammation similar to the chronic inflammation observed in EB; 3) The blister plane is at the level of the lamina lucida in the Basement Membrane Zone (BMZ) for both JEB and SM-induced injury. This suggests that injury induced by vesicants is not unique and probably involves malformation of laminin 332. Understanding the role of laminin 332 in SM induced blisters may provide perspectives for future molecular therapeutic countermeasures against SM exposure.

Introduction

Sulfur mustard (bis-2-chloroethyl sulfide, HD, SM) is a potent vesicant that has been used as a chemical warfare agent since War World I (WWI). The threat still exists, and SM was used by ISIS as a terrorist agent as recently as last year and reported to the United Nations Security Council in May, 2017. Other mustard agents including nitrogen mustard (bis-2-chloroethyl methylamine, HN2, NM) and half mustard (2-Chloroethyl Ethyl Sulfide, CEES) are less potent structurally related analogs of sulfur mustard, but still are able to cause severe chemical burns. The primary organ targets of mustard agents include the skin, eyes, and respiratory tissues [1,2]. Since SM rapidly penetrates human skin and can cause extensive chemical burns after a latency period of 2-24 hours. Depending on the dose, the vesicant used, and the exposure time, other symptoms include edema and erythema. Vesication and the appearance of pale yellow fluid-filled blisters usually occurs 10-48 h post exposure [3]. These microbullae may coalesce into larger bullae and blisters may last for days to weeks after exposure [4,5]. Chronic effects of SM-induced skin injury may last for months, and even lead to persistent pigmentation changes with severe scarring for years [6,7]. Since SM is so highly reactive it can form adducts and readily cross links DNA, proteins, lipids and other nucleophilic molecules in cells [8,9]. SM may also disrupt tissue structure by altering extracellular molecules such

as laminin 332, the major component of the anchoring filaments, organelles that directly attach the basal keratinocytes to the anchoring fibrils located in the dermis [10]. Due to its close proximity to the basement membrane zone; disruption of laminin 332 can cause gross separation of the epidermis from the dermis. SM-induced cutaneous injury is accompanied by a prolonged and massive inflammatory response which leads to a delay in wound repair and may result in severe scarring [11,12]. Victims of SM skin injury frequently require hospitalization for months. To date, there are no FDA-approved therapies for cutaneous mustard injury. Current treatments include blister aspiration, physical debridement, irrigation, topical antibiotics, and sterile dressings which relieve symptoms, prevent infection, and promote healing [7]. There continues to be an active search for therapies and countermeasures to minimize SM-induced cutaneous damage and to promote wound repair. Since it is prohibited to test therapeutic agents against vesicants on human subjects, SM research studies on animal models have been used to partly clarify the mechanism of action of vesicants.

Animal models (e.g., the Mouse Ear Vesicant Model (MEVM) and Hairless dorsal Mouse Skin Model (HMSM) were developed as reasonable alternatives to human studies [13-18]. In murine models, SM only induces microbullae and microblisters rather than large fluid-filled blisters as observed in humans [14,18]. However, there are similar histopathologic changes between human and murine skin, including detachment of the epithelium. Sulfur mustard induces severe inflammation and histopathological damage as illustrated in the MEVM study shown in Figure 1. Mouse ears were treated with SM on the ventral (anterior surface) side of the ear. Pyknotic nuclei of the basal keratinocytes are visible shortly after exposure. There is an influx of inflammatory cells and pronounced edema evident within the first 24 h post SM exposure of the treated ears. Significant swelling (as an index of edema) of the tissue continues beyond 24 h. This is reflected by the increased distances between the ventral (anterior surface) and dorsal (posterior surface) sides of the ear and by abundant clear areas in the dermis (Figure 1, panels A-D; note that these panels are identical magnifications). Pronounced inflammatory cell infiltration to the dermis (inflammatory cell nuclei stained purple) commenced within one day and continued to progress until 7 days post exposure. Necrosis was commonly observed in mouse ear skin from the treated side by 72-168 h. The injury continues to expand and eventually including the untreated, dorsal side (exterior surface). The severity of skin wound of the dorsal untreated side showed a temporal lag time slightly behind the ventral or treated side [17,19].

3.1. Cutaneous basement membrane zone and laminin 332

The cutaneous basement membrane is composed of independent but associated networks of type IV collagens and laminins, into which proteoglycans and fibronectin are intercalated. It is now clear that epithelial laminins are a genetically and functionally distinct laminin subclass comprised of laminin 311, 321 and 332 ($\alpha3\beta1\gamma1$; $\alpha3\beta2\gamma1$; $\alpha3\beta3\gamma2$). Small amounts of additional laminins (laminin 111 -$\alpha1\beta1\gamma1$, laminin 411 -$\alpha4\beta1\gamma1$, and laminin 511 -$\alpha5\beta1\gamma1$) are also present but their function is unknown. Evidence indicates that laminin 332 is essential to epithelial-basement membrane stability as it is the key bridging molecule between the keratinocyte hemidesmosomal integrin $\alpha6\beta4$, and the anchoring fibril protein collagen VII. This function allows laminin 332 to form a continuum of molecules that originates within the epithelial cell, extends into the underlying extracellular matrix, and functions to reinforce cell-matrix attachment [20,10]. This attachment helps maintain the integrity of skin to externally applied forces. Laminin 332 is composed of three individual polypeptides that are separate gene products [21]. A schematic diagram of laminin 332 is shown in Figure 2.

The three disulfide-linked polypeptide chains (α3, β3, and γ2) are assembled in the Endoplasmic Reticulum (ER) into a cross-shaped heterotrimer and held together by an α-helical coiled-coil structure [22]. Two of the chains (α3 and γ2) are proteolytically processed to modify the function of laminin 332. Laminin 332 is an important ligand in the adhesion of keratinocytes. It is part of an interconnecting protein structure termed the "anchoring complex" that is required for keratinocyte attachment to the underlying dermis.

3.2. Histological similarity of SM induced blisters and epidermolysis bullosa (EB)

The anchoring complex is an ultrastructural feature of the skin [20]. It is a component of a string of molecules that originates within the epithelial cell, extends into the underlying ECM, and functions to reinforce cell-matrix attachment. This attachment helps maintain the integrity of skin against externally applied forces. There are many structural proteins involved in these interactions, including keratins, Bullous Pemphigoid Antigens (BPAG), laminin is of orms, and type VII collagen [23,24].

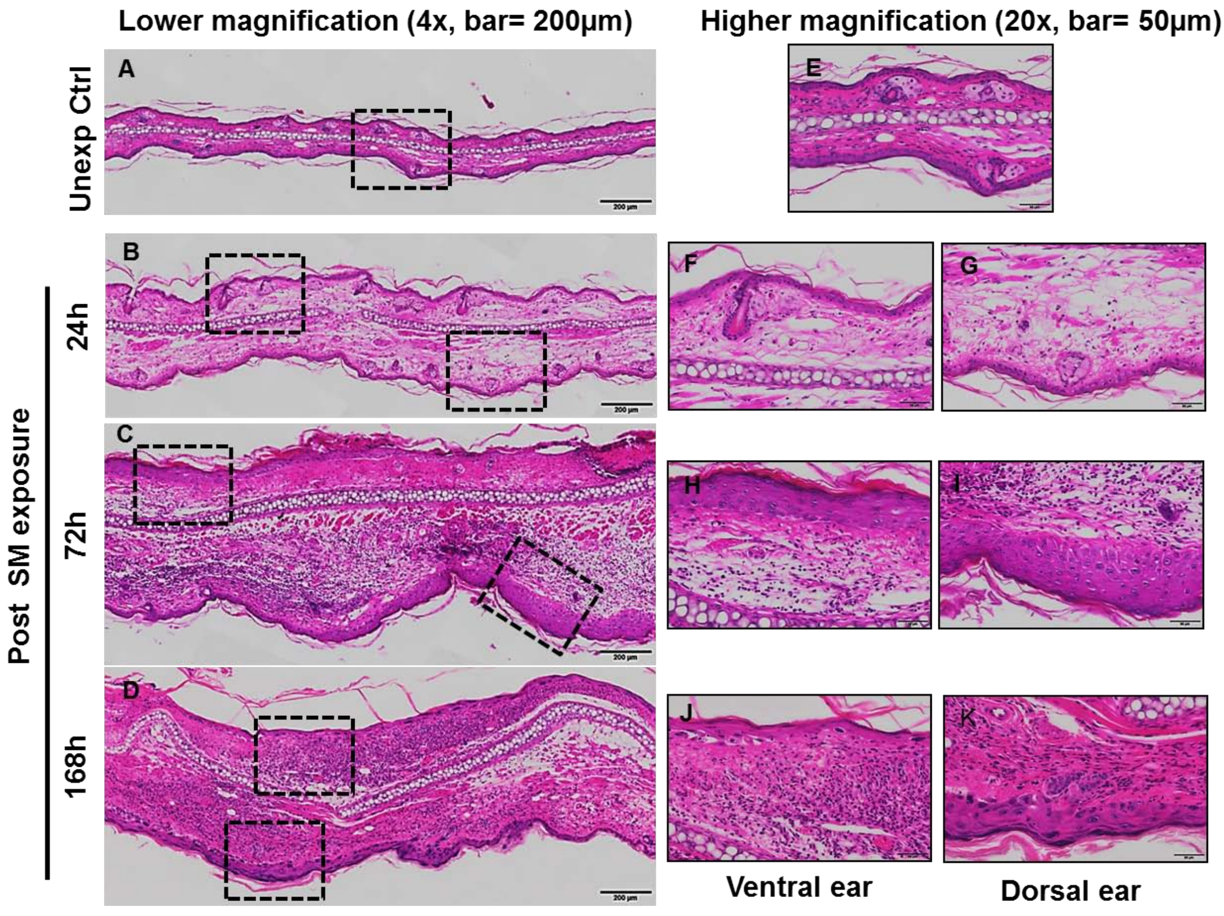

Figure 1: Histopathological structural changes of sulfur mustard exposed skin injury in a time course MEVM study. Hematoxylin and eosin stained histology of mouse ear skin in the MEVM. Tissues were collected 24, 72, and 168 h post-SM exposures. Bar represents 200μm (in, 4X lower magnification panels A, B, C, D). The rectangular dotted line boxes are shown in higher magnification for ventral ear and dorsal ear. Bar represents 50μm (in, 20X higher magnification panels E, F, G, H, I, J, K) The ventral (interior, SM treated side) ear is the upper surface, and the dorsal (exterior, untreated side) is the lower surface. The ventral and dorsal side of ear skin is separated by cartilage. Panels (A) and (E): untreated control skin showed tight dermal-epidermal junction, dense dermal tissue and regular appearance of sebaceous glands and hair follicles on both sides of ear skin. Panels (B, F, and G) 24 h, (C, H, and I) 72 h, and (D, J, and K) 168 h, are images of SM treated ear skin. All SM-exposed ear skin (including SM treated (ventral) and untreated (dorsal) sides of skin) demonstrate edema as indicated by the increased skin thickness. Increasing inflammatory cell infiltration over time was apparent in SM skin wounds (purple stained dots to be maximum at 168h post SM exposure).

If the attachment of any of these proteins is disrupted, the skin, which is fragile, can pull loose from the underlying dermis. This is true for EB, a group of skin diseases characterized by fragility and easy blistering of the skin in response to mechanical trauma. The clinical severity and extent of tissue involvement in EB are highly variable, as is reflected in the extensive and primarily descriptive nomenclature chosen for these conditions in the past [25-28]. Clinical, morphological, and ultrastructural observations have been used to classify inherited EB into three major categories based on the level of blister formation within the skin [29] the simplex (EBS), junctional (JEB), and dystrophic (DEB) forms.

There are similarities between SM-induced skin injury and EB. It was reported that 4 to 6 hours after accidental exposure to sulfur mustard, the victim had skin pathology that involved targeting of epidermal basal cells, disabling of hemidesmosomes, and recruitment of inflammatory cells similar to that seen in EB syndrome [30]. Another study demonstrated EB-like separation of the epidermis and dermis in mouse ears treated with SM [14]. This separation caused by SM exposure is most similar to the junctional epidermolysis bullosa. JEB is an autosomal recessive skin blistering disease with both lethal and nonlethal forms [31]. As with SM exposure, in JEB, the skin blistering occurs at the level of the lamina lucida and involves the hemidesmosomal proteins that attach basal keratinocytes to the Basement Membrane Zone (BMZ) [25]. One of the major glycoproteins of the lamina lucida that anchor the keratinocytes is laminin 332 which is made up of three individual polypeptides that are separate gene products [21]. These genes have been named LAMA3 [32]; LAMB3 [20] ; and LAMC2 [33,34]. Their molecular weights are α3; 200 kDa; β3; 145 kDa, and γ2; 155 kDa [35]. There has been extensive mutation detection analysis in JEB patients and multiple mutations in any of these three separate polypeptide genes have been shown to cause JEB (α3, [36]; β3, [37]; γ2, [38]. In fact, multiple mutations have been found in all of these genes. Another JEB gene mutation has been reported for collagen XVII (also called BPAG2 or BP 180) [39]. Collagen XVII is a hemidesmosomal protein that binds one end of laminin 332. Ultrastructural evidence of SM-induced basal cell BMZ damage has been documented for both laminin 332 and bullous pemphigoid 180 (collagen XVII) [31,14].

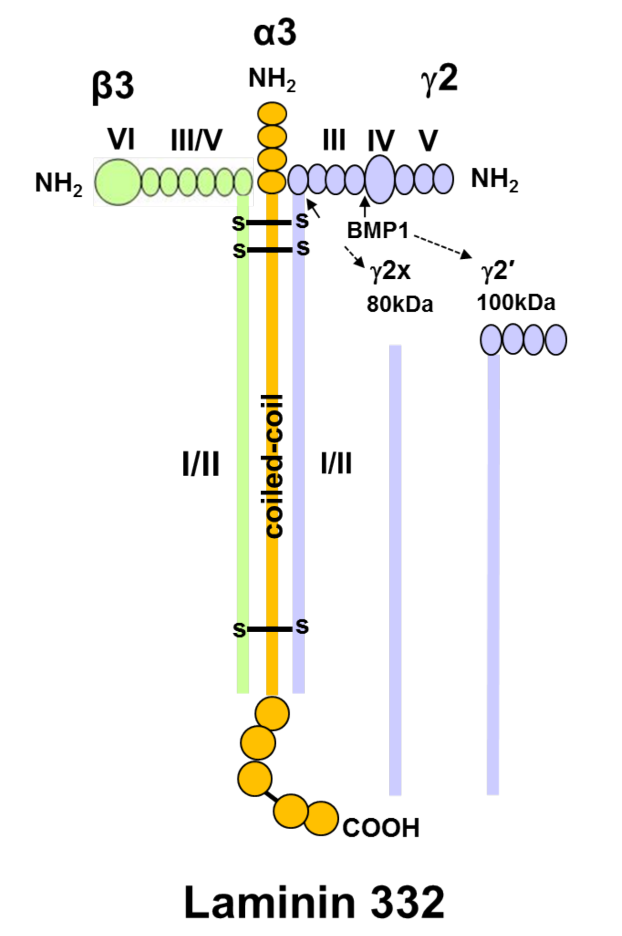

Figure 2: Schematic diagram of laminin 332. Laminin 332 is composed of three individual polypeptides (with separate gene products) which are disulfide-linked together into a cross-shaped heterotrimer and held together by an α-helical coiled-coil structure. Protein domains are labeled in Roman numerals (I - VI). Two of the chains (α3 and γ2) are proteolytically processed to modify the function of laminin 332. Laminin γ2 can further be processed by proteases (e.g., BMP1) at various sites (pointed by arrows) into fragments (e.g., γ2x, and γ2′). The monomeric short arm of Laminin γ2 functions as substrate for cell migration.

3.3. Proteases activation in EB and SM induced blisters

Morphological similarities between EB- and SM-induced skin injuries are not the only evidence of similar pathologies. Proteases are elevated in cutaneous inflammatory responses [40] and are known to play key roles in the disruption of connective tissue proteins and other basement membrane proteins [41,14]. Some of the same proteases that are released or induced because of inflammation, tissue damage, or cell death may be involved in both processes because their morphological outcomes are so similar. Skin fibroblasts from patients with EB have been reported to have abnormally high levels of collagenase and/or stromelysin [42,43]. The simplex form of EB has been shown to express high levels of gelatinases (MMP-2 and MMP-9) [44,45]. Proteases also are implicated in other subepidermal blistering diseases, such as bullous pemphigoid [46], dermatitis herpetiformis [46], and pemphigus vulgaris [47]. Increased protease activity also has been reported following SM exposure in vitro using human Peripheral Blood Lymphocytes (PBL) [48] or human epidermal keratinocytes [49,50], and ex vivo in rabbit skin organ cultures [51,52] and human skin explants [53], and in vivo in hairless guinea pig skin [54]. While elevation of MMP9 mRNA expression was observed in a weanling pig model at 24h post SM exposure [55]. In a time course study using the MEVM showed protease induction on both the gene and protein level for MMP9, an early marker of skin injury 24-168 hr post SM exposure. This suggests that MMP9 may be one of the proteases identified to play a role in both SM induced and JEB induced skin injury [16]. Because proteases are likely to be involved in the pathophysiology of SM induced blistering, the effectiveness of protease inhibitors as therapeutic agents against SM skin damage should be investigated. In fact, several reports show some success in the use of protease inhibitors both in vitro in cell culture [56,15], and in vivo in a mouse model [57-59]. The use of bi-functional anti-inflammatory compounds also show efficacy in reducing MMP9 expression in SM induced skin wound [60].

3.4. Role of laminin 332 in SM induced skin injury and wound repair

Laminin 332 is required for final attachment of the basal keratinocytes to the dermis below in normal healthy skin and fully repaired tissue. It is bifunctional in that it not only anchors keratinocytes to the dermis [61], but acts as a substrate for the migration of keratinocytes over a wound bed during repair of injured skin [62]. SM-induced alkylation may disrupt both functions of laminin 332.

Keratinocytes are known to secrete proteases (eg., BMP1, MMP19) that cleave the γ 2 chain of Laminn 332; while others such as MMP2 and MMP14 cleave Laminin α 3 [9,63]. Post SM exposure, mRNA expression of all three chains (α3, β3, and γ2 chains) of laminin 332 was reduced at 6-24h post SM exposure. mRNA expression of Lamininα3 and β3 chains were back to basal level by 72-168 h post exposure. But the laminin γ2 chain mRNA was dramatically increased at 72-168 h post exposure in mouse skin wounds [19]. If translation is directly proportional to the transcriptional amount of mRNA, this suggests there may be preferential de novo synthesis of lamininγ 2 polypeptide compared to the other two chains. Over expression of laminin γ 2 has been reported as a potential marker of invasion of metastatic cancers and epithelial cell migration during wound repair. Western blot analysis showed several fragments of laminin γ 2 proportionally increased with time after SM treatment in mouse ear skin at 72-168 hr post exposure. This small processed form of laminin γ 2 preferentially increased and may be associated with normal keratinocyte migration over a wound site. Nevertheless, SM damaged blisters often lead to delayed wound repair. This prompts the question whether the increased processed form of laminin γ 2 facilitates keratinocyte migration for wound healing.

The epidermal basal cells are the primary target for the cross linking of DNA after SM exposure [64]. The cytotoxicity and DNA damage response by the alkylating agents are too overwhelmed to properly repair the affected cells and they ultimately undergo cell death [65]. Under extreme cellular damage, the ERS response is activated when normal ER function is perturbed (e.g., accumulation of malfolded proteins, lipid imbalances or excessive ionic changes in the ER lumen) [66]. Skin exposed to SM may also result in homeostatic dysfunction to trigger the endoplasmic reticulum stress (ERS) response in order to increase ER capacity for proper protein folding [67]. Confocal microscopy experiments showed expression of BiP (ERS marker) and laminin γ2 for 72h SM injured skin, suggesting there was accumulation of laminin γ2 in the ER (Figure 3).

required for normal skin repair. Firstly, the secretion of abundant processed laminin γ2 may be critical in promoting cell migration for proper wound repair.

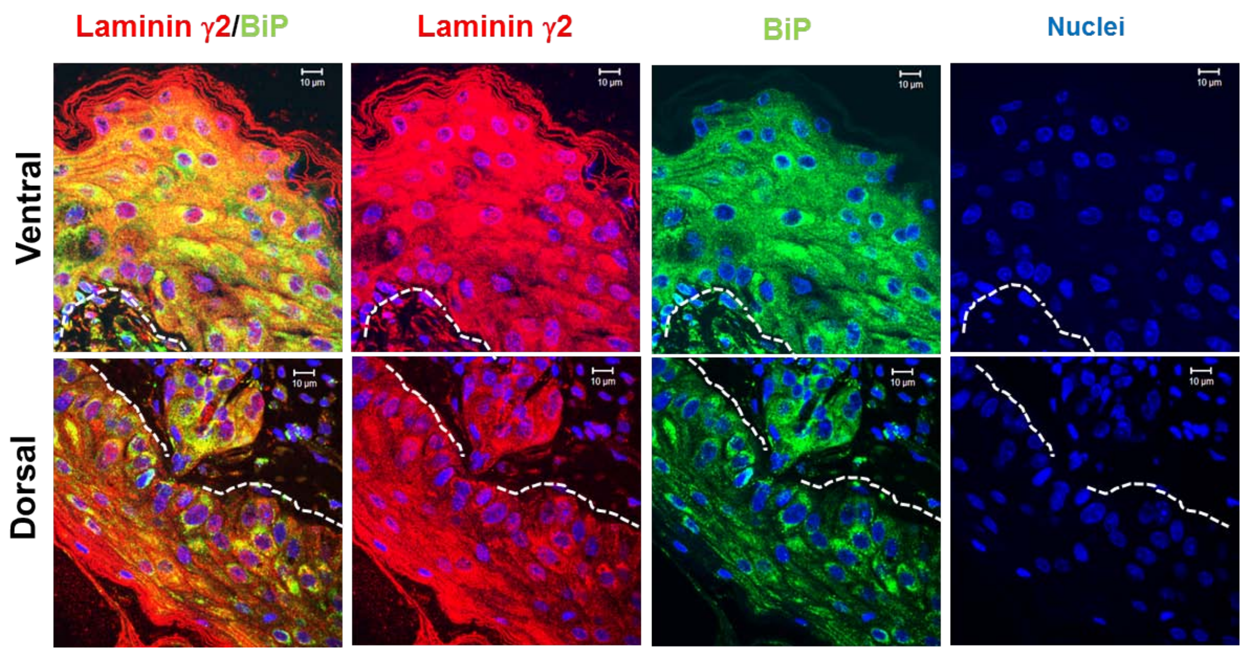

Figure 3: Dual immunofluorescent labeled laminin γ2 (red) and BiP/GRP78 (green, an ER stress marker). Panels illustrate The ERS response to SM damage in mouse skin at 72h post exposure. Projected confocal microscopy show preferential coexpression of laminin γ2 and BiP/GRP78 (yellow) in the suprabasal epithelium for both the ventral and dorsal murine ear skin. The epidermal/dermal basement membrane zone marked as dotted lines. Scale bar is 10μm.

It appears that the accumulation of laminin γ2 in the ER, is, specifically localized in the migrating, but not in proliferating cells. It is intriguing that, similar to invasive basal carcinoma cells, laminin γ2 is found in the migrating keratinocytes that have left the cell cycle, but not in the proliferating cells [68]. Excess production of laminin γ2 has been used as a potential marker for invasion of malignant and metastatic cancers; likewise, it may potentially present as biomarker in SM induced blisters. Further studies are needed to elucidate the role of preferentially expressed and processed laminin γ2 monomers and their influence on migration of epidermal keratinocytes injured by SM.

4. Summary

Laminin 332 plays dual functions in SM induced skin wound repair: Cell migration and cell adhesion are

Secondly, the expression of all three chains of laminin 332 is required to allow the proform to be secreted into the ECM where it is assembled into the anchoring filaments and attaches to collagen XVII in the hemidesmosomes on one end and collagen VII in the anchoring fibrils on the other end. Furthermore, the severe tissue damage by SM may activate ERS response for cell survival. Enhancing ERS survival pathways may help to facilitate proper folding and assembly of laminin 332 for proper wound repair.

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