Heparanase Inhibitors Facilitate the Assembly of the Basement Membrane in Artificial Skin

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Abstract: Recent research suggests that the basement membrane at the dermal-epidermal junction of the skin plays an important role in maintaining a healthy epidermis and dermis, and repeated damage to the skin can destabilize the skin and accelerate the aging process. Skin-equivalent models are suitable for studying the reconstruction of the basement membrane and its contribution to epidermal homeostasis because they lack the basement membrane and show abnormal expression of epidermal differentiation markers. By using these models, it has been shown that reconstruction of the basement membrane is enhanced not only by supplying basement membrane components, but also by inhibiting proteinases such as urokinase and matrix metalloproteinase. Although matrix metalloproteinase inhibitors assist in the reconstruction of the basement membrane structure, their action is not sufficient to promote its functional recovery. However, heparanase inhibitors stabilize the heparan sulfate chains of perlecan (a heparan sulfate proteoglycan) and promote the regulation of heparan sulfate binding growth factors in the basement membrane. Heparan sulfate promotes effective protein-protein interactions, thereby facilitating the assembly of type VII collagen anchoring fibrils and elastin-associated microfibrils. Using both matrix metalloproteinase inhibitors and heparanase inhibitors, the basement membrane in a skin-equivalent model comes close to recapitulating the structure and function of an in vivo basement membrane. Therefore, by using an appropriate dermis model and suitable protease inhibitors, it may be possible to produce skin-equivalent models that are more similar to natural skin.

Keywords: Basement membrane, heparan sulfate, heparanase, inhibitor, matrix metalloproteinase, three-dimensional culture.

INTRODUCTION

The skin is composed of three distinct layers: the subcutaneous tissue, the dermis, and the epidermis. The epidermis is the uppermost or epithelial layer of the skin. It contains several cell types, including keratinocytes, melanocytes, Langerhans cells, and Merkel cells. The dermis lies between the epidermis and subcutaneous tissue and is mainly composed of fibroblasts, but also contains other cell types such as blood vessel cells, mast cells, and immune cells. The subcutaneous tissue is the fat layer below the dermis, and this contains cell types such as adipocytes, neurons, and blood vessel cells. Groups of cells within organs are surrounded by an extracellular matrix (ECM). The ECM provides the mechanical framework for each tissue and organ, is a substrate for cell signaling, supports organogenesis, and helps to maintain homeostasis [1]. In the skin, there are various types of extracellular matrix proteins, including collagens, elastins, and proteoglycans. Collagens are the most abundant component of the extracellular matrix. The dermis, for example, mainly contains type I and type III collagens, but also includes types V, VI, XII, and XIV [2].

For researchers seeking to understand cell proliferation and differentiation in the skin, one approach is to use an in vitro cell culture system that simulates the biochemical and mechanical signals between the dermis and epidermis mediated by the basement membrane (BM), thereby regulating tissue development [3]. However, the basement membrane at the interface of the dermis and epidermis is a critical component of a skin model that has been very difficult to recreate. Approaches to creating a model of the epidermal and dermal layers with an active basement membrane is the focus of this review.

SKIN-EQUIVALENT TISSUE ENGINEERING

In an in vitro tissue engineering system, cells are usually seeded onto three-dimensional polymer scaffolds that require
an ECM composition suited for the attachment and growth of keratinocytes and promotes physical stability. An in vitro reconstructed skin model composed of the epidermal and dermal layers has been used as a model of skin constitution. Therefore, in these models, the layers are composed of skin fibroblasts, extracellular matrix, and keratinocytes. Bell et al. reported that a contracted collagen lattice containing fibroblasts might serve as a substrate for epidermal cells (Bell model) [4] and also presented a functional full-thickness skin model designed as a graft to cover open skin wounds or burned skin. In this dermal model, the proliferation of fibroblasts is inhibited similarly to the in vivo condition, and the fibroblasts are restricted in an environment of retracted collagen [5]. The model has been useful for exploring the dynamics of the BM [6, 7] and for studies of epidermal differentiation, dermal–epidermal interaction, and tumor cell invasion [8, 9]. However, its use is limited because a reduction in the final surface area of the skin equivalent (SE) can take place without a peripheral anchorage [10]. Skin model equivalents have been developed using many other methods that will not be covered here in detail (Table 1). These models are distinguished from each other by how the dermal layer is created. The quality of the dermal equivalent should allow for the development and maintenance of a pluristratified and differentiated epidermis firmly anchored by an organized dermal-epidermal junction [11].

Keratinocytes are seeded onto the dermal layer and then cultured at the air-liquid interface, a condition that induces epidermal differentiation. Type I and III collagens are present in the reconstructed dermis, whereas type IV and VII collagens, as well as laminin, are deposited at the dermo-epidermal junction [12]. Conversely, epidermal quality may influence the properties of cells in the dermis [13]. For example, crosstalk between the epidermis and the dermis plays an important role in maintaining homeostasis of the skin [11, 14]. In addition to keratinocytes and fibroblasts, it is thought that melanocytes, vascular endothelial cells, neurons, and Langerhans cells in the skin might also be under the influence of such crosstalk [11, 15-19]. Therefore, the BM at the interface of the epidermis and dermis is critical for proper skin function [20].

**BASEMENT MEMBRANE**

The epidermal BM at the dermo-epidermal junction (DEJ) is mainly composed of type IV and Type VII collagens, several laminins (such as 332, 331, and 511), nidogen, and perlecan [21]. The anchoring fibrils, which anchor the BM to the underlying dermis, are composed primarily of type VII collagen [22]. The BM also contains a lamina densa (a sheet-like structure mainly composed of type IV collagen [23]) and a lamina lucida (the region between the lamina densa and the basal surface of the basal keratinocytes). It forms electron-dense plaques known as hemidesmosomes that contain at least two transmembrane proteins, α6β4 integrin and collagen type XVII (BP180). Hemidesmosomes link the epidermis to the BM [24, 25]. Perlecan, a heparan sulfate proteoglycan (HSPG) with three heparan sulfate chains, is a structural constituent of the BM at the DEJ [26]. The epidermis is structurally joined to the BM through hemidesmosomes and collagen type VII anchoring fibrils.

Disruption of the BM results in skin fragility and skin diseases such as epidermolysis bullosa [27]. Past studies have shown that gene abnormalities related to laminin 332, α6β4 integrin, BP180, and collagen type VII may be the cause of such issues [28-32] and they have highlighted the importance of the structural integrity of the interface between the epidermis and the dermis. Iriyama et al. reported that the heparan sulfate chains of perlecan were degraded by active heparanase in UVB-irradiated human skin and SEs, causing skin damage consistent with photoaging [33].

Proteases such as metalloproteinases and the plasmin-plasminogen system are involved in normal and aberrant cutaneous wound repair. Some proteases also activate growth factors and other proteases in wounds, and regulate growth factor signaling by shedding growth factor receptors on the cell surface. Furthermore, proteases break down the

| Table 1. Various methods of dermal model creation. |
|-----------------------------------------------|
| **Polymer scaffolds** | **Cell Types** | **Additives** | **Culture period** | **Ref.** |
| 1 | Animal collagen I | fibroblast | no | 3~ 4days | [4] |
| 2 | Collagen sponge | fibroblast | no | 4 weeks | [133-135] |
| 3 | Esterified hyaluronic acid fibers | fibroblast | TGF-beta | 6 weeks | [136] |
| 4 | Matrix complex (type I and III collagens and chondroitin sulfate) | fibroblast | no | 3 weeks | [137, 138] |
| 5 | No exogenous scaffolding elements | fibroblast | no | 4 weeks | [139-141] |
| 6 | Fibronectin | fibroblast | no | 7 days | [142] |
| 7 | De-epidermized derma | - | no | - | [143] |
ECM produced by cells [34], an effect that should be recapitulated in a skin model. Therefore, in this review, I discuss how the combined use of protease inhibitors in a skin model culture can affect the reconstruction of the BM and the function of a skin model.

THE ADHESION OF THE EPIDERMIS AND THE DERMIS IN SEs

To generate a typical SE model, human keratinocytes are seeded onto a contracted collagen gel containing dermal fibroblasts, and specific culture conditions are used to ensure that the model is highly similar to native tissue [35]. These models are an important tool for the study of cell-cell, cell-matrix, and dermal-epidermal interactions, and for pharmacological testing. However, currently, human SEs have a limited lifespan of approximately eight weeks, rendering them unsuitable for long-term studies. Further, the structural integrity of the interface joining the dermis and epidermis is fragile. Therefore, studies requiring a skin model are typically based on non-human dermal equivalents, e.g., collagen originating from rat tendons, bovine tissues, and pigs' skin. Therefore, these alternative non-human biopolymers allow for the rapid formation of SEs [36].

De-epidermized dermis (DED, Table 1) may offer a human alternative to animal derived dermal equivalents, but the application of this method is limited by uncontrollable variation in DED thickness and low availability of the native tissue. Other dermal equivalents (Table 1) can be generated by using a primary human fibroblast-derived dermal matrix with, for example, chitosan-cross-linked collagen-glycosaminoglycan. In these methods, cultivation must be maintained for weeks in order to form a dermal model. Keratinocytes are then seeded onto the dermal model, leading to the formation of the epidermis structure (this process is common to all methods), which takes a week. The most important factor for success is a strong connection between the dermis and the epidermis through the BM, similar to that in the living body. Additionally, the communication between the formed epidermal and dermal layers should be normal and continually maintained. Dynamic interactions and the cooperation of many components, from fibroblasts to keratinocytes, are required to create a skin-specific ECM, and the BM dermo-epidermal junction is vital. The BM has been reported to play an important role in the attachment of the epidermis, the regulation of cellular polarity, and the survival, proliferation, and differentiation of cells [37-40].

Type I, III, and V collagens and fibrillin-1 are derived from fibroblasts, whereas the components of the hemidesmosome, BP180 and integrin β4, are derived from keratinocytes [41]. However, tenascin-C, type IV and VII collagens, perlecan, and nidogen are synthesized by both fibroblasts and keratinocytes. Many components produced by both fibroblasts and keratinocytes are required for the formation of a normal epidermis and BM in SEs, and factors secreted by keratinocytes activate matrix metalloproteinases (MMPs).

Previous studies have demonstrated that treatment with the synthetic MMP inhibitor CGS27023, which blocks degradation of type IV collagen, leads to the formation of a continuous lamina densa in SEs [42]. This suggests that a collagen degradation system is active in SE models. In previously described three-dimensional skin models, the lamina densa and hemidesmosomes were visible in some areas after two weeks, and anchoring fibrils were organized after six weeks of culture [43]. Marionnet et al. detected expression of BM components after just a few days of culture, and immunostaining revealed that BM proteins were at the DEJ after eight days of culture [44]. These reports suggest that long cultivation times are needed to form the basement membrane, even if the expression and deposition of basement membrane components were detected early. In other words, in SE models that lack a BM at the early stage of culture, a balance in the production and degradation of matrix proteins affects protein assembly and the higher-order structure of the BM.

UVB exposure to skin is known to increase the synthesis of MMPs, urokinase-type plasminogen activator (uPA), uPAR, and heparanase, which degrades BM components such as type VII and IV collagens, laminin 332, and perlecan [33, 45-48]. SE models can partially mimic the photo-aging process because BMs are missing at the DEJ and large amounts of MMP (including gelatinases MMP-2 and MMP-9), active uPA, and uPA are present in the conditioned SE media [7, 42].

Additionally, a plasmin inhibitor, aprotinin, can restore the assembly of the BM at the DEJ following damage caused by the addition of plasminogen [48]. Laminin 332 is also capable of enhancing BM assembly [6]. Battaglia et al. showed that HSPG promoted assembly of the BM by interacting with components of the BM, such as laminins [49]. Furthermore, the deposition of collagen VII is polarized to the basal side by the addition of an MMP inhibitor, and treatment with a combination of MMP inhibitor and heparanase inhibitor enhances the formation of anchoring fibrils that bind to the lamina densa. Sher et al. reported that keratinocyte-derived cell perlecan is essential for the survival of the regenerative cell population required to form a multilayered epidermis [50]. Together these findings suggest that if a uniform lamina densa with anchoring fibrils that bind to the basal lamina is formed, a typical SE can be produced that firmly connects dermis, epidermis, and BM.

THE ROLE OF HEPORAN SULFATE IN ELASTIC FIBER FORMATION IN THE SE

Elastic fibers, which consist of polymerized tropoelastin monomers surrounded by a mantle of microfibrils (MFs) (reviewed in [51]), undergo normal age-related changes, leading to loose skin, stiff vessels, and increased blood pressure. In living skin, mature elastin fibrils are distributed among collagen bundles. In electron micrographs, elastic fibers appear as an amorphous core of elastin surrounded by 12-nm diameter MFs containing the glycoprotein fibrillin. The primary roles of elastic fibers include providing elasticity and recoil in tissues and organs and maintaining structural integrity against mechanical strain over a lifetime [52]. Moreover, elastic fibers, such as oxytalan fibers in papillary dermis, are associated with not only skin resilience, but also skin surface texture [53, 54]. Yamazaki et al. reported that elastin-free oxytalan MFs originating from the DEJ are continuous with elaunin-type MFs (with a small amount of
elastin monomers prior to cross-linking by lysyl oxidase (LOX) [56, 57].

Casasco et al. studied elastin expression using a SE with a dermis model made of a collagen gel [58]. They reported that, after about two weeks of culture, high-resolution immunocytochemistry and electron microscopy could be used for specific labeling and detection of the elastin-immunoreactive material in the ECM. However, the distribution of immunoreactive material was small and it was not assembled into fibers. They also reported that a well-developed rough endoplasmic reticulum was visible in the fibroblasts of the dermal model. Duplan-Perrat et al. also investigated elastin formation in a dermal model made of a sponge made of collagen, chitosan, and GAGs. The expression of elastin in the SE was demonstrated 30 days after culture (15 days after the seeding keratinocytes). Using electron microscopy, elastin could be seen only after 45 days (30 days after keratinocyte-seeding). Clearly, the formation of elastin requires long time periods [53]. The authors suggested that MFs first assembled at the DEJ without elastin, and then elastin was deposited on the hollow-shaped MF bundles beneath the DEJ, and finally, true elastic fibers, combining microfibrilar and amorphous components, formed [59]. It has also been reported that the maturation of elastic fibers is affected by keratinocytes [60]; the synthesis and assembly of fibrillin into MFs concomitant with basal lamina formation favored the regular perpendicular distribution of oxytalan fibers at the DEJ in SE models. It was also reported that the presence of keratinocytes and ECM generated by fibroblasts in the porous structures of the dermis were needed to trigger elastin deposition on MFs [61].

In natural skin, mature elastin fibers are distributed between mesh-like layers of collagen fibers. The main components of elastin fibers are amorphous elastin and MFs, but they also contain many other proteins such as tropoelastin, fibrulin, fibrillin, nidogen, laminin, perlecan, and LTBP [62-65]. Several groups have reported that heparan sulfates are associated with elastin-related proteins [66-71]. Tiedemann et al. demonstrated that the proteoglycan perlecan is an associated with MFs in close proximity to BM zones and revealed interactions between perlecan domains I/II and a central region of fibrillin-1. They also reported that perlecan null embryos had fewer MFs at the DEJ when compared with wild-type littermates [72]. Ritty et al. reported that interactions between heparan sulfate and fibrillins play a critical role in fibrillin network assembly and are important for matrix deposition by fibroblasts [73]. Sabatier et al. suggested that heparan sulfates could play a role in stabilizing the growth of MFs, and could assist in the process of elongation and interactions with other MF-associated proteins [71]. Based on these reports, the presence of perlecan with heparan sulfate is expected to play an important role in elastin formation. Iriyama et al. reported that when the heparanase inhibitor (1-[4-(1H-benzoimidazol-2-yl)-phenyl]-3-[4-(1H-benzoimidazol-2-yl)-phenyl]-urea (BIPBIPU) [74]) and a matrix metalloproteinase inhibitor were applied to a SE, regeneration and repair of the dermal and epidermal BM was significantly enhanced [75]. Additionally, a previous study showed that elastin-like structures similar to the elastin reported by Rodrigo et al. was observed in a skin model only after cultures were treated with both a heparanase inhibitor and MMP inhibitor (Fig. 1) [76, 77].

Together, these results suggest that a more stable artificial skin model could be produced in relatively short-term culture if a suitable combination of inhibitors that influence the structure and composition of the extracellular matrix were identified and characterized.

HEPARANASE

Heparan sulfate specific endo-beta-D-glucuronidase activity in normal tissue was first reported by Höök et al. in 1975, and today the enzyme is known as heparanase [78, 79]. Heparanase is well studied in cancer research because it is a useful marker for tumor metastasis. The heparan sulfate side chains of the proteoglycan scaffold in the ECM can be digested by heparanase. The enzyme is implicated not only in
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The function of HSPGs in the basement membrane

Heparan sulfate is the most abundant of the GAG family, and is present through the epidermis [99, 100]. It is a linear polysaccharide chain composed of D-glucosamine, D-glucuronic acid, and L-iduronic acid [101]. The largest part of each GAG chain contains a number of repeating disaccharide units composed of alternating glucuronic acid and N-acetylgalactosamine, which during biosynthesis are subjected to a range of modifications, including O-sulfation at various positions, N-deacetylation, N-sulfation of N-acetylgalactosamine residues, and C-5 epimerization of glucuronic acid to iduronic acid. Theoretically, up to 48 different disaccharides could occur in heparan sulfate because of these modifications [102]. Therefore, it is possible that a large number of sequences exist, and such structural diversity underlies the diversified biological functions of GAGs.

HSPGs consist of a protein core to which heparan sulfate side chains are covalently attached. These complex macromolecules are highly abundant in the ECM and are thought to play an important structural role, contributing to ECM integrity and insolubility [103]. HSPGs are involved in the regulation of molecular transfer through the BM, protection of matrix components such as type IV collagen from proteolytic attack, storage of various cytokines and growth factors, and are key molecular players in inflammation [94, 104].

Heparanase contributes to angiogenesis because HSPGs are cleaved by heparanase, releasing angiogenic factors such as fibroblast growth factors (FGFs), vascular endothelial growth factors A (VEGF-A), heparin growth factors, platelet-derived growth factors, and transforming growth factor-β [93, 105-114]. Since heparan sulfate side chains produce a large negative charge, HSPGs create a diffusion barrier, as well as an anchoring port. For example, heparan sulfate regulates the activity of FGFs; heparan sulfate-binding growth factors, by acting as coreceptors on the cell surface, enhance FGF receptor affinity for FGF [110, 115], providing a storage reservoir in the BM [102, 108, 116-118]. Heparan sulfate also regulates angiogenesis via its interaction with VEGF-A. Kiryushko et al. showed that signaling through S100A4, part of the S100 family, depends on interactions with HSPGs at the cell surface [119]. Thus, GAGs may act as coreceptors for S100 proteins in neurons [119-121]. Furthermore, heparanase-induced loss of heparan sulfate at the DEJ has been implicated in the process of pigmentation in human skin. Specifically, proliferation and differentiation of melanocytes is regulated by various factors, including leukemia inhibitory factor (LIF), stem cell factor (SCF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), and granulocyte-macrophage colony stimulating factor (GM-CSF). Because HGF and bFGF bind to heparan sulfate at the DEJ, these growth factors are restricted from reaching melanocytes in normal conditions [20, 122-124]. Therefore, taken together, these studies show that HSPGs are essential for several important biological processes.

The role of HSPGs in DEJ organization in the SE

The von Willebrand factor domain, including the NC-1 domain of type VII collagen, interacts with heparin and proteoglycans [125]. Therefore, an increase in heparin sulfate chains in the presence of heparanase inhibitor promotes effective interaction between the NC-1 domain of type VII collagen and the beta 3 chain of laminin 332 at the DEJ, facilitating the assembly of anchoring fibrils. Iriyama et al. showed that the addition of a heparanase inhibitor, together with an MMP inhibitor, promoted anchoring fibril structure formation in the BM in a SE model [126]. Pinozou et al. showed that synthesis of heparan sulfate proteoglycans such as syndecan and perlecan (a mixed heparan sulfate/chondroitin sulfate proteoglycan localized at the skin BM zone) was induced by a xylopyranoside derivative, which
also increased GAG synthesis. In addition, a xylopyranoside derivative increased the expression of BM components such as type IV and VII collagens, procollagen-1, laminin 332, and fibrillin-1 in human skin [127]. These reports suggest that the increase in HSPGs impacts DEJ organization by decomposition, suppression, or synthetic induction of heparan sulfate side chains.

Sahuc et al. used electron microscopy to show that linear deposition of type VII collagen was detected in immunohistochemical studies after two weeks of culture, and anchoring fibrils, a continuous lamina densa, and numerous hemidesmosomes were detected where keratinocytes were in direct contact with fibroblasts [128]. Breitkreutz et al. observed BM formation during skin regeneration in human keratinocyte transplants in nude mice. They showed that the deposition of type VII collagen became continuous and BM-anchoring fibrils developed at 22 days, indicating final tissue consolidation [129]. Although the formation of the basement membrane takes time in the living body, the use of appropriate enzyme inhibitors and/or protein production enhancers, such as MMP inhibitors, heparanase inhibitors, and GAG synthesis inducers could lead to the faster deposition of BM components and assist in the maturation of BM structure. By using heparanase and MMP inhibitors, it was possible to create a typical SE that closely resembles the structure of natural skin in a relatively short period (~4-14 days) [75].

In addition to accelerating the maturation of BMs, heparanase and MMP inhibitors also indicate that presence of active fibroblasts in the dermis of artificial skin. In SEs, similar to natural skin, collagen synthesis, protein secretion, and cellular production of ECM and fibers is active. Moreover, in the dermis of SEs cultivated using MMP inhibitors and heparanase inhibitors, the formation of higher-order structures of fibroblasts, including fibripositors and rough endoplasmic reticulum, have been observed (Fig. 2). Fibripositors are actin-rich plasma membrane protrusions that secrete and deposit collagen fibrils. Moreover, this phenomenon has only been observed with embryonic fibroblasts [130-132]. Fibroblasts with fibripositors are considered active and have been observed beneath the BM, close to the epidermis. However, the mechanism underlying the formation of fibripositors and collagen secretion from fibroblasts, which is induced by MMP and heparanase inhibitors, is currently unknown. Further studies are needed to understand the mechanisms controlling dermal tissue formation and the influence of the epidermis and basement membrane.

**SUMMARY**

One approach to understanding the mechanism of cell proliferation and differentiation is the in vitro study of three-dimensional cell cultures that simulate the biochemical and mechanical signals that regulate tissue development. The functional BM at the interface of the epidermis and the dermis is important for epidermal differentiation. In addition to type IV and VII collagens, which are important for the formation of BMs, the heparan sulfate chains of proteoglycans such as perlecan give functionality to the BM. The use of appropriate enzyme inhibitors and/or protein production enhancers could promote BM production and speed wound healing in living tissue. Additionally, the artificial skin model is a useful tool for investigating the crosstalk that exists between the epidermis and the dermis.

**CONFLICT OF INTEREST**

The author confirms that this article content has no conflict of interest.

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