Matrix Metalloproteinase Gelatinase B (MMP-9) Coordinates and Effects Epithelial Regeneration*

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We studied the role of the matrix metalloproteinase gelatinase B (gelB; MMP-9) in epithelial regeneration using the gelB-deficient mouse. We report the novel finding that, in contrast to other MMPs expressed at the front of the advancing epithelial sheet in wounds of cornea, skin, or trachea, gelB acts to inhibit the rate of wound closure. We determined this to be due to control of cell replication, a novel capacity for MMPs not previously described. We also found that gelB delays the inflammatory response. Acceleration of these processes in gelB-deficient mice is correlated with a delay in signal transduction through Smad2, a transcription factor that inhibits cell proliferation, and in accumulation of epithelial-associated interleukin-1α, a cytokine that inhibits Smad2 signaling and promotes the inflammatory response. GelB-deficient mice also reveal defects in remodeling of extracellular matrix at the epithelial basement membrane zone, in particular, failure to effectively remove the fibrinogen provisional matrix. We conclude that gelB coordinates and effects multiple events involved in the process of epithelial regeneration.

Epithelial tissues surface all organs of the body exposed to the external environment, acting as a protective barrier. Unlike most organ stromal tissues, epithelia can regenerate completely following injury. Epithelial regeneration involves multiple distinct processes, including the concerted migration of cells as a sheet to resurface the wound bed, mitosis to replace cell numbers, re-stratification of the sheet into a multilayered structure, and restoration of stable adhesive interaction with the underlying tissue stroma (1–6). These processes must be temporally coordinated with one another, and with the protective inflammatory response, which also occurs in response to injury. This is thought to involve an orderly progression and synergism of cytokines and growth factors within the wound environment (7–15). Despite many recent advances, mechanisms for integrating and orchestrating the multiple processes involved in epithelial regeneration are still poorly understood.

The matrix metalloproteinases (MMPs) are a family of zinc endopeptidases that act as key effectors and regulators of tissue remodeling in vertebrates (16). Molecular substrates for the MMPs include all classes of extracellular matrix proteins and molecules that organize tissues such as the cadherins (17, 18). MMPs are also reactive against signaling molecules such as cytokines and growth factors, controlling their activity and bioavailability (18). While MMP activity is regulated at multiple levels, gene expression constitutes the major control mechanism (19). Induced expression of an array of MMPs occurs as part of the tissue response to injury (20, 21). Inappropriate expression or overexpression of MMPs contributes to the pathophysiology of diverse disorders occurring across all organ systems (22), including healing disorders (20, 23–25).

Gelatinase B (gelB; MMP-9) is an MMP that catalyzes cleavage of denatured collagens of all types and native basement membrane components (26, 16). In addition, recent work has identified fibrinogen (27), α1-proteinase inhibitor (79), interleukin-1 (IL-1) (28, 29), and transforming growth factor-β (TGF-β) as gelB substrates (30). GelB is not expressed in the normal cornea, skin, or trachea; however, expression is induced along with a number of other MMPs in cells at the front of the migrating epithelial sheet as it begins to resurface the wound bed following injury (22, 31–33). Inflammatory cells infiltrating the wound bed also produce gelB (34, 35). GelB expression in the epithelium spreads progressively distal to the migrating front once wound resurfacing is complete and persists for several weeks thereafter (32, 36). The timing correlates with the period during which provisional wound matrix is resorbed and new structures for epithelial/stromal adhesion are assembled in the epithelial basement membrane zone (37). Overexpres-
corneas using a Summit Apex Excimer Laser (Summit, Waltham, MA) and protein concentration was determined with the Bio-Rad reagent. Methacrylate cross-sections (6 μm) were processed for indirect immunofluorescent labeling with a monoclonal antibody against BrdU (Becton Dickinson, San Jose, CA) following the manufacturer’s instructions. Color development was accomplished by incubation with horseradish peroxidase-conjugated secondary antibody followed by chemiluminescence (PerkinElmer Life Sciences, Boston, MA). The entire corneal epithelium was then removed by gentle scraping with a scalpel. The pooled tissues from 10 eyes were lysed in a buffer containing 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each aprotonin, leupeptin, pepstatin, 1 mM Na2VO4, 10 mM NaF. Equal amounts of protein (10 μg/sample) were separated by 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes and probed with specific primary antibody reagents. Immunodetection of primary antibody binding was accomplished by incubation with horseradish peroxidase-conjugated secondary antibody followed by chemiluminescence (PerkinElmer Life Sciences) and exposure to x-ray film. Rabbit anti-Smad3 and anti-phospho-Smad2 were purchased from Upstate Biotechnology (Lake Placid, NY), and monoclonal anti-Smad2 (clone 18) was purchased from Transduction Laboratories (San Diego, CA). A goat polyclonal antibody raised against recombinant mouse IL-1α (catalog number AF-400-NA) was removed from R & D Systems (Minneapolis, MN). Information in the company catalogue states that this antibody was selected for its ability to neutralize the biological activity of recombinant mouse IL-1α. Based on direct enzyme-linked immunosorbent assay and Western blot results, this antibody showed less than 10% cross-reactivity with recombinant human IL-1α. Additionally, indirect enzyme-linked immunosorbent assays this antibody showed no cross-reactivity with other cytokines tested.

RESULTS

GelB-deficient Mice Have Normal Corneas—Mice homozygous for a targeted mutation that inactivates the gene for gelB show delayed growth of long bones due to impaired vascularization of the growth plate, but ultimately they attain normal adulthood (38). Routine gross examination of adult eyes revealed no obvious anatomic differences between gelB-deficient homozygous and their normal counterparts. Comparison of the gelB-deficient mice were of normal size and length, with no obvious differences compared to normal controls. Examination of the anterior corneal surface with a slit lamp showed no evidence of keratoconus. GelB-deficient mice were of normal size and length, with no obvious differences compared to normal controls. Examination of the anterior corneal surface with a slit lamp showed no evidence of keratoconus.
Increased Rate of Corneal Re-epithelialization in GelB-deficient Mice—To investigate the effects of gelB deficiency on regeneration of the corneal epithelium following injury, we used an epithelial debridement model (42, 32) adapted for the mouse in which the epithelium is surgically removed to the level of the basement membrane within a circular region of 1.5-mm diameter. We collected epithelial tissue from the repairing portions of normal and gelB-deficient corneas at a time when wounds were partially closed, and analyzed tissue extracts by gelatin zymography (Fig. 1A). A gelatinase of a size appropriate to be the gelB proenzyme was detectable in extracts derived from littermate control mice, but was absent from extracts derived from gelB-deficient mice. In contrast, a gelatinase of the size appropriate to be the gelatinase A (gelA; MMP-2) proenzyme was detectable in extracts from both gelB-deficient mice and their normal counterparts. The intensity of the gelA band was essentially the same in both mouse lines, indicating that gelB deficiency does not result in compensatory up-regulation of this related enzyme.

A time course experiment was performed over the period of wound closure, and the rate of re-epithelialization was compared between gelB-deficient mice and normal littermate controls. Fig. 1B shows representative images of the remaining epithelial defects at a time when wounds were partially closed. No difference was observed in the quality of re-epithelialization between mouse strains. Corneas from both gelB KO mice and their normal counterparts re-epithelialized without incident, with no gross evidence of inflammation. In contrast, the rate of re-epithelialization was faster in the gelB-deficient mice and their normal counterparts; however, re-epithelialization was still significantly faster in the gelB-deficient mice (Fig. 1D).

To learn whether faster wound closure generalizes to other organs, we examined the rate of healing of an excisional wound in the skin (Fig. 1E). As in cornea, wound closure in skin was found to occur significantly more rapidly in gelB-deficient mice than in their normal control counterparts. These results indicate that the findings in cornea are not tissue-specific.

The GelB-deficient Re-epithelialization Phenotype Is Due to a Direct Loss-of-function in the Eye and Is Different from the Phenotype Resulting from Broad Spectrum MMP Deficiency—To differentiate between systemic and local factors in the gelB-deficient phenotype, we repeated the surgical debridement experiment, but eyes were enucleated following surgery and placed in organ culture for healing. The difference in the re-epithelialization rate between normal and gelB-deficient mice was less than when re-epithelialization occurred in the mouse in situ, but was still significant at 15 h (p < 0.002; n = 10; data not shown) and 20 h after surgery (Fig. 2A). These data indicate that at least some of the effects of gelB deficiency are attributable to local factors.

To provide evidence that the corneal re-epithelialization phenotype in the gelB-deficient mice is due to a direct loss-of-function, we attempted a rescue experiment by adding purified gelB protein to the corneas of gelB-deficient mice undergoing re-epithelialization in organ culture. GelB was added in the latent proenzyme form, because this is the form that predominates in re-epithelializing debridement wounds (see Fig. 1A). Our expectation was that the exogenously added proenzyme would be converted to an active form through the same extracellular mechanisms utilized for activation of endogenously produced enzyme during wound repair. At 1 μg/ml of exogenously added gelB proenzyme, the retardation of re-epithelialization rate was significant (Fig. 2B). In other words, exogenous addition of gelB returned the rate of wound re-epithelialization in the gelB-deficient mouse back toward
normal, consistent with rescue of the deficiency phenotype. These data support the conclusion that the gelB-deficient re-epithelialization phenotype results from a direct loss-of-function due to inactivation of the gene for gelB.

Inhibition of broad-spectrum or specific MMP activity has been reported to inhibit epithelial cell migration in culture (21, 33, 43) and to retard re-epithelialization of skin wounds in organ culture and in vivo (21, 44). To learn whether this is also true in our corneal model, we examined the effects of ilomastat, a broad spectrum MMP inhibitor (39, 45) on re-epithelialization in normal mice. While the $K_i$ for gelatinases determined in test tube assay is 0.4 nM, inhibition of MMPs in tissues requires much higher concentrations; we used the dose range determined to be effective by Chin and Werb (46) in their studies of tumor invasion by confocal microscopy on corneal whole mounts, taken 18 h after surgery. Arrows indicate the migrating epithelial front. The arrowhead indicates invading inflammatory cells in the wound bed. Immunofluorescence, frozen cross-sections of corneas taken 16 h after surgery and stained by indirect immunofluorescence using the inflammatory cell marker Mac-1. Shown is the central portion of cornea which is still not re-epithelialized at this time point.

Earlier Inflammatory Cell Infiltration, Increased Deposition of Provisional Matrix, and Enhanced Rate of Epithelial Cell Replacement in GelB-deficient Mice—Tangential optical sectioning by confocal microscopy on corneal whole mounts stained with rhodamine-phalloidin revealed no differences between gelB-deficient mice and their normal counterparts in the overall appearance, or in organization of the actin filaments, in cells at the advancing epithelial front (Fig. 3, Confocal, arrows). However, in the course of these experiments we observed the presence of inflammatory cells in the wound bed of gelB-deficient mice (Fig. 3, Confocal, $+/+$, arrowhead), that were not seen in their normal counterparts (Fig. 3, Confocal, $+/-$, arrowhead). Absence of gelB does not appear to alter the capacity of inflammatory cells to respond to a chemotactic stimulus, extravasate from blood vessels, or accumulate in tissues (47). However, a deficiency in gelB was shown to delay the resolution of the contact hypersensitivity response in skin (48). A systematic investigation of inflammatory cell infiltration by immunofluorescent microscopy on cross-sections revealed consistent staining with the Mac-1 marker in corneas from gelB-deficient mice examined prior to epithelial closure (16–18 h). Confocal, tangential optical sections by confocal microscopy through the epithelium of rhodamine-phalloidin-stained corneal whole mounts, taken 18 h after surgery. Arrows indicate the migrating epithelial front. The arrowhead indicates invading inflammatory cells in the wound bed. Immunofluorescence, frozen cross-sections of corneas taken 16 h after surgery and stained by indirect immunofluorescence using the inflammatory cell marker Mac-1. Shown is the central portion of cornea which is still not re-epithelialized at this time point. Arrowheads indicate positively stained cells. Corneas were counterstained with Hoechst222 dye (DAPI) which binds the nuclear DNA of all cells. H & E, corneal cross-sections taken 18 h after surgery were stained with hematoxylin and eosin and visualized by light microscopy. The presence of eosinophilic deposits in the gelB-deficient cornea is indicated by the arrowheads. The leading edge of the migrating epithelium is indicated by the arrows. BrdU, corneal cross-sections stained with BrdU antibody to visualize cells undergoing DNA synthesis. Arrows indicate positively stained cells.

In histologically stained cross-sections, the migrating epithelial sheet appeared as a monolayer in normal mice but was multilayered in the gelB-deficient mice (Fig. 3, H & E, arrows). In addition, an eosinophilic deposit was apparent in the wound bed and below the migrating epithelium in some gelB-deficient mice that was not observed in their normal counterparts (Fig. 3, H & E, arrowheads). This seemed likely to represent an accumulation of provisional wound matrix and is discussed further below. Bromodeoxyuridine (BrdU) labeling was per-
formed on corneal cross-sections to identify cells undergoing DNA synthesis (Fig. 3, BrdU). It is known that epithelial injury stimulates proliferation of cells distal to the wound edge, while the rate of proliferation decreases in cells at the migratory front (49, 50). Consistent with this, BrdU-labeled cells from corneas of both normal and gelB-deficient mice were concentrated in the peripheral epithelium, with fewer labeled cells localized to the migratory front (central). In both locations, however, the number of labeled cells was clearly greater in the gelB-deficient mice. These data provide evidence that gelB deficiency enhances the rate of epithelial resurfacing of corneal wounds by increasing the pressure exerted as a result of cell proliferation.

**Epithelial Regeneration in GelB-deficient Mice**

GelB Deficiency Is Associated with a Delay in Smad2 Signaling and an Increase in Cell-associated IL-1α in the Regenerating Corneal Epithelium—GelB can cleave a variety of molecules involved in cell signaling (18), and thus gelB deficiency might alter the net signaling information received at the cell surface and cause the changes observed above. A deficiency in the transcription factor Smad3 was recently shown to enhance the rate of cutaneous wound re-epithelialization by increasing cell proliferation (51), similar to our findings here on the gelB-deficient phenotype. Both Smad3 and its closely related homologue, Smad2, translocate from the cytoplasm to the nucleus once phosphorylated in response to signals received at the cell surface (52). Immunohistochemical staining of cross-sections through corneas of normal mice revealed that Smad3 undergoes translocation into nuclei throughout the regenerating corneal epithelium following debridement surgery (Fig. 4A). Western blotting revealed that Smad2 and Smad3 activity was clearly increased in the regenerating epithelium of normal mice (Fig. 4B, +/+). For Smad2, increased activity was demonstrated by comparison of the levels of total Smad2 and phosphorylated Smad2. For Smad3, reprobing of the same Western blot revealed a new immunoreactive band of slower electrophoretic mobility in regenerating epithelium, consistent with phosphorylation. These findings are consistent with a role for Smad2 and Smad3 in controlling the rate of epithelial cell proliferation.

Western blot analysis of the regenerating epithelium from gelB-deficient mice showed the same overall changes in Smad2 and Smad3 as the normal mice (Fig. 4B, −/−). The amount of the putative active form of Smad3 was similar in the gelB-deficient mice and their normal counterparts. However, activation of Smad2 was clearly delayed in the gelB-deficient mice; an increase was not observed until the 16-h time point after debridement surgery. Sections were immunostained with antibody to Smad3. Arrows indicate translocation of Smad3 protein into nuclei in the migrating epithelium. B, Western blot of cell lysates from uninjured corneal epithelium (0 h), and from the migrating epithelium of corneas, 8 h (8 h) and 16 h (16 h) after epithelial abrasion surgery. Lanes were loaded with equal amounts of protein as determined by BCA assay, and equality of loading was confirmed by Coomassie Blue staining on a parallel set of lanes. The blot was probed with antibodies against Smad3, Smad2, and with an antibody specific for the phosphorylated form of Smad2 (pSmad2). Immunoreactive proteins are indicated by an arrow. The putative phosphorylated Smad3 isoform (pSmad3) is also indicated by an arrow. C, Western blot of cell lysates from uninjured corneal epithelium (0 h), and from migrating epithelium collected 8 h (8 h) after epithelial abrasion surgery. The blot was probed with an antibody to IL-1α. The immunoreactive protein is indicated with an arrow.

**Epithelial Regeneration in GelB-deficient Mice Compromises Corneal Transparency**—In normal skin or corneal wounds, a provisional extracellular matrix composed of fibrin(ogen) and fibronectin is deposited in the wound bed from the serum or tear film, and provides a substrate for cell attachment and migration (57–62). This matrix is resorbed once the wound is resurfaced and is replaced by new epithelial/stromal anchoring complexes (37, 64). The eosophinophil deposits apparent in the wound bed of gelB-deficient mice (see Fig. 3, H & E), suggested that remodeling in the basement membrane zone might be defective. We investigated this hypothesis by immunofluorescence analysis (Fig. 5A). In the unwounded areas of both gelB-deficient and normal corneas that had been healing for 6 days following PRK, the anchoring fibril component laminin-5 was present as a thin linear band between the epithelium and the stroma, and there was no staining for fibronectin or fibrinogen. This staining pattern had returned in the wound bed of the normal control mice, consistent with the timing of basement membrane zone remodeling observed in previous studies (32, 60). In contrast, the immunoreactive band of laminin-5 was thick and uneven in the wound bed of gelB-deficient corneas at the 6-day time point, and small amounts of fibronec-
Photographed 2 or 6 days after PRK. Indirect immunofluorescence microscopy was used to identify reactive fibrin(ogen) which accumulates in eyes from gelB-deficient mice. The results indicate corneal opacities. A, cross-sections from corneas taken from normal (+/+ and gelB-deficient mice (−/−) 6 days after PRK. Indirect immunofluorescence microscopy was used to identify laminin-5, fibronectin, and fibrin(ogen). The arrow indicates immunoreactive fibrin(ogen) which accumulates in eyes from gelB-deficient mice. B, eyes of normal mouse (+/+ and gelB-deficient mice (−/−) were photographed 2 or 6 days after PRK. Arrows indicate corneal opacities.

DISCUSSION

MMP expression is induced in cells at the migrating epithelial front in healing wounds of cornea, skin, or lung, and the combined evidence to date (including data presented here) has supported the hypothesis that MMPs function to promote epithelial resurfacing by stimulating cell migration (22, 43, 44). Moreover, there is evidence to support this role specifically for gelB (33, 65). MMPs, including gelB, have further been implicated in a larger way in the process of cell migration involving many other tissues. Again, the roles identified for MMPs have been consistently facilitative and include the clearing of extracellular matrix to break down physical barriers, modulation of adhesive interactions with the extracellular matrix to release cells and to provide traction for their movement, and exposure of signals necessary to effect motor function and provide chemotraction (18). The results reported here reveal that, while gelB may be involved in epithelial sheet migration, it is clearly not essential for this process in the normal in vivo situation. In fact, we show that a deficiency in gelB actually accelerates the rate of normal wound resurfacing. This is the first time that an MMP has been shown to exert negative control over cell migration. We show that gelB does this by inhibiting cell replication in the migrating epithelial sheet. This is a novel role for an MMP, not previously identified.

Peripheral to the main purpose of the current study, we made the new and significant observation that Smad2/Smad3 signaling is activated in the regenerating corneal epithelium. Smad signaling inhibits cell replication (52, 66), and thus Smad activation seems at first counterproductive to the requirement for cell replacement in epithelial regeneration. This can be understood, however, when we take into account that epithelial regeneration involves several other distinct processes, including resurfacing of the wound bed, re-stratification into a multilayered structure, and restoration of stable adhesive interaction with the underlying stroma. Each of these processes is associated with a withdrawal from the cell cycle (49, 50), and it is here that Smad activation may play a role.

Significantly, we found a specific delay in activation of Smad2 but not Smad3 in the gelB-deficient mice. Smad2 and Smad3 are the major downstream effectors of TGF-β signaling; however, there is accumulating evidence to suggest that these proteins are functionally different (52, 66). While activity of both are stimulated by TGF-β signaling, they can be differentially regulated as a result of cross-talk among signaling pathways activated by other extracellular ligands. The IL-1 signaling pathway interacts with the Smad signaling pathway in this way; therefore the premature increase in IL-1β levels observed in this study may translate into a specific delay in Smad-2 signaling (76–78, 80–81). GelB is selectively active against the IL-1β isofrom, although a minor capacity to cleave IL-1α could still translate into a major effect in a specific microenvironment in situ. However, GelB could alter the accumulation of IL-1α by

**TABLE I**

Comparison of corneal clarity in repairing corneas of gelB-deficient mice and their normal counterparts following photorefractive keratectomy.

| Mouse strain | Haze grading |
|--------------|--------------|
|              | Day 2 | Day 6 | Day 14 |
| Normal       | 0 (0/12) | 0 (0/12) | 0 (0/12) |
| GelB-deficient | 0.5 (2/12) | 1 (10/12) | 2 (8/12) |

Corneal clarity was graded by an observer unaware of the mouse genotype. Grade 0 indicate complete clarity with no sign of haze, grade 0.5 indicate mild haze, grade 1 indicates well defined diffuse haze, grade 2 indicates obstruction of iris detail, and grade 3 indicate complete obstruction of the anterior chamber and iris. The number in parentheses indicates the number of animals with observable haze/the number of total animals examined. p ≤ 0.001 (comparing normal and gelB-deficient mice using the non-parametric Mann-Whitney U test).
any number of indirect mechanisms including degradation of a proteinase that degrades IL-1α or degradation of a cytokine that stimulates expression of IL-1α by epithelial cells (for example, see Refs. 27, 30, and 79). Therefore, it seems likely that the levels of IL-1α may be controlled by gelB through the enzymes action against multiple substrate.

Our findings also indicate that gelB controls the timing of the inflammatory response in the repairing cornea. Again, this may be attributed to premature accumulation of IL-1α, which is a chemoattractant for leukocytes (63). In a similar vein, an alteration in cytokine profiles was identified as the mechanism for delayed inflammatory cell resolution in a cutaneous chemical hypersensitivity model applied to the gelB-deficient mice (48). The joint regulation of wound closure and inflammation by controlling the levels of a single cytokine, IL-1α, could serve to coordinate the timing of these two processes.

Our work shows that gelB alters the “instructions” that cells receive from the microenvironment, thus mediating very specific alterations in intracellular signaling pathways, and fine tuning of the regenerative process. Not only must cell replication be temporally-coordinated with the other processes involved in epithelial regeneration, but also spatially coordinated. Thus, the progressive change in expression pattern of gelB during epithelial regeneration may be a key factor in its ability to perform a fine tuning function (31, 32, 36). Expression at the leading edge of the migrating corneal epithelium may serve to coordinate what is happening at the migrating front with the cell replication that occurs distal to the front. Later, when the wound is closed, expression across the entire regenerating epithelium may be important for the complete resorption of provisional matrix and restoration of normal epithelial/stromal adhesion.

The accumulation of fibrin(ogen) in the wound bed was a striking aspect of the gelB-deficient phenotype found in this study and indicates that gelB acts not only to coordinate but also to affect events involved in epithelial regeneration. Recent work has shown that pericellular fibrinolysis by migrating endothelial cells is MMP-mediated (67). As this article was in preparation, it was reported that fibrin(ogen) accumulation occurs in the gelB-deficient mouse in a kidney disease model (68) and can be visually detected within days, it persists in chronic cutaneous wounds (75, 74). It has been suggested that excessive fibrin deposition may contribute to pathophysiology, by “trapping” of cytokines controlling epithelial cell dynamics, and by physically interfering with the restoration of stable epithelial/stromal adhesion.

Taking the findings of this study into consideration with the results of previous work, we conclude that a balance of gelB activity must be struck for health of epithelial tissues; too little activity or too much activity can both lead to pathology. However, it appears that timing and location of gelB expression in the microenvironment is also critical to the overall picture of health or pathology. Understanding the mechanisms for controlling the pattern of gelB expression will be a future challenge.

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