Novel Laminin 5 γ2-chain Fragments Potentiating the Limbal Epithelial Cell Outgrowth on Amniotic Membrane

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Purpose. Matrix metalloproteases (MMPs)-mediated extracellular matrix (ECM) degradation potentially releases cryptic motility factors involved in somatic stem cell migration and epithelial outgrowth. The authors previously demonstrated that MMP-9 is upregulated in limbal epithelial cells cultivated on amniotic membrane (AM). Here, the authors further investigated whether plasminogen activator (PA)/plasmin regulates MMP-9 activity in this model implicated in the processing of laminin 5 (Ln5), a component of amniotic basement membrane.

Methods. Limbal epithelial cells migrated from limbal explants were expanded on intact AM. The activities and proteins of uPA and MMP-9 in limbal epithelial cells were determined by fibrin and gelatin zymography, reverse transcription–polymerase chain reaction (RT-PCR), Western blotting, and immunofluorescent staining. Specific pharmacological inhibitors including MMPs inhibitor GM6001, MMP-2/-9 inhibitor, and uPA inhibitor B428 were used to determine whether the PA/plasmin/MMP-9 axis induces cell growth via Ln5 in this model.

Results. These data showed that MMP-9 activity was attenuated by a selective uPA inhibitor, B428. Furthermore, MMP-9 activity was enhanced by exogenous addition or pre-incubation with plasmin. These results demonstrated that PA/plasmin regulates MMP-9 expression. An interesting proteolytic fragment of Ln5 γ2-chain was suppressed by pretreatment with GM6001, B428, or neutralizing antibodies of MMP-9 and uPA, indicating that Ln5 γ2-chain is processed by uPA/MMP-9. Moreover, the extent of limbal outgrowth was also retarded by B428.

Conclusions. This study suggested that MMP-9 activity was upregulated by PA/plasmin, which in turn processed Ln5 γ2-chain to facilitate limbal outgrowth on intact AM. (*Invest Ophthalmol Vis Sci.* 2009;50:4631–4639) DOI:10.1167/iovs.08-3060

Amniotic membrane (AM) transplantation has been applied for a continuously widening spectrum of ophthalmic indications that are effective in reconstructing the ocular surface in the setting of persistent corneal epithelial defects,[1] corneal ulcerations,[2] and partial or total limbal stem cell deficiency (LSCD).[3–5] The mechanisms underlying AM transplantation action include promoting re-epithelialization, anti-inflammation, anti-fibrosis, anti-angiogenesis, and anti-apoptosis.[6,7] Recently, ex vivo cultivation and expansion of a small limbal explant has been successfully performed in reconstructing the ocular surfaces of patients with LSCD by using AM as a supporting matrix,[4,5] suggesting that AM is not only a good scaffold for ocular surface reconstruction but also provides a suitable microenvironment for expansion of limbal epithelial cells.

Degradation of ECM by two distinct pathways, the matrix metalloproteinase (MMP) pathway[8] and the plasminogen activators (PAs)/plasmin proteolytic axis,[9] has been implicated in many physiological and pathologic processes. MMPs belong to a family of zinc-dependent endopeptidases that digest ECM components, and PAs are serine proteases originally described as matrix- and fibrin-degrading enzymes, including urokinase-type PA (uPA) and tissue-type PA. uPA can catalyze the conversion of plasminogen to plasmin and has been shown to play a dominant role in the control of matrix integrity through intracellular signaling. uPA also modulates various biological behaviors, including cell adhesion, migration, and tissue regeneration.[9,10] On the other hand, the primary role of tissue-type PA is to generate plasmin in the presence of fibrin.[11]

Pro-uPA is activated by plasmin, which regulates a self-maintained feedback mechanism of pro-uPA and plasminogen activation.[12] Several chemical compounds and proteinases, including plasmin, trypsin, tissue kallikrein, or cathepsin G are capable of activating MMPs in vitro.[13,14] Previous studies have demonstrated that plasmin can activate several MMPs in vitro.[15] Moreover, in vivo study reveals that expression of MMPs may be stimulated by uPA.[15] The generation of plasmin provides an efficient activity directed to the degradation of ECM components which are similar to those of human AM, such as fibronectin and laminin 5 (Ln5).[16,17] Ln5, an $\alpha3\beta3\gamma2$ heterotrimer glycoprotein, mediates cellular adhesion,[18] hemidesmosome formation,[19] and cell migration.[20,21] Recent studies have demonstrated that MMP-2,[20] membrane type 1 (MT1)-MMP,[21] and several other MMPs, can process the Ln5 γ2-chain to reveal a cryptic migratory signal inducing epithelial

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cell migration.[20,21] These studies imply that MMPs-regulated epithelial cells migration might be mediated through processing the γ2-chain of Ln5.

In our previous study, we found that MMP-9 plays an important role in the interaction of cell-cell matrix in ex vivo expansion of human limbal epithelial cells on AM.[22] Herein we further investigated whether the expression and activity of PA/plasmin proteolytic enzymes lead to MMP-9 expression and their roles in processing Ln5 γ2-chain in this model. Our results demonstrated that MMP-9 activity was upregulated by PA/plasmin, which processed Ln5 γ2-chain to facilitate limbal outgrowth on intact AM.

Materials and Methods

Human Limbal Explants Cultured on Human AM

Human corneoscleral buttons from either penetrating keratoplasty or fresh cadaveric globes were obtained from the Eye Bank (Chang Gung Memorial Hospital, Tao-Yuan, Taiwan) and handled according to the tenets of the Declaration of Helsinki. The time lapse between procurement of the limbal tissue and setting up explant culture was within approximately 48 hours. The tissue was rinsed three times with DMEM/F-12 containing 50 μg/mL gentamicin and 1.25 μg/mL amphotericin B. After careful removal of excess sclera, iris, corneal endothelium, conjunctiva, and Tenon's capsule, the remaining tissue was placed in a culture dish and cut into cubes of approximately 1.5 × 2 × 3 mm with a scalpel. Human AM was procured by elective cesarean section from Chang Gung Memorial Hospital (Keelung, Taiwan) with properly informed consent and was processed as described.[1] The AM was thawed and placed on a culture dish with the basement membrane side up and incubated at 37°C in a humidified incubator under 5% CO_2 and 95% air overnight. The preparation of limbal explant culture on intact AM was as previously described.[7] Briefly, a limbal explant was placed on the center of the AM and cultured in a medium made of an equal volume of HEPES-buffered DMEM containing bicarbonate and F-12 and supplemented with 5% FBS, 0.5% dimethyl sulfoxide, 2 ng/mL mouse epidermal growth factor (EGF), 5 μg/mL insulin, 5 μg/mL transferrin, 5 ng/mL selenium, 0.5 μg/mL hydrocortisone, 30 ng/mL cholera toxin, 50 μg/mL gentamicin, and 1.25 μg/mL amphotericin B. Cultures were incubated at 37°C under 5% CO_2 and 95% air. Cultures at the end of the second week in H/A^{intact} group were treated with or without 20 μM B428 (generously provided by Murray J. Towle, Eisai Research Institute, Andover, MA). The conditioned media were changed and saved every 2 to 3 days for gelatin and fibrin zymographic analyses of MMPs and uPA. The extent of limbal outgrowth was monitored with a phase-contrast microscope.

Gelatin and Fibrin Zymographic Analysis

For zymographic analysis of MMP, the culture medium was loaded onto a 10% SDS-PAGE containing 1 mg/mL gelatin; for zymographic analysis of uPA, the culture medium was loaded onto a 12% SDS-PAGE containing 1.2 mg/mL bovine fibrinogen, 0.1 NIH unit/mL bovine plasminogen solution, and 1 unit/mL bovine thrombin. After electrophoresis, gels were washed twice with 2.5% Triton X-100 for 30 minutes at room temperature to remove SDS followed by washing twice with 50 mM Tris-HCL buffer for 30 minutes to recover enzyme activity, and then incubated in MMP developing buffer (50 mM Tris, 40 mM HCl, 200 mM NaCl, 5 mM $CaCl_2$, and 0.2% Briji) and in uPA reaction buffer (30 mM Tris, pH 7.4, 200 mM NaCl, and 0.02% NaN_3) at 37°C for 16 hours on a gyratory shaker, respectively. After incubation, gels were stained in 30% methanol, 10% acetic acid, and 0.5% (wt/vol) Coomassie brilliant blue for 1 hour followed by destaining with methanol/acetic acid/water (100/150/750, v/v/v). Nonstaining bands representing the levels of MMP and uPA were measured by spot density measurement using a digital imaging analysis system (UN-SCAN-IT Version 6.1; Silk Scientific, Orem, UT).

In Situ Gelatin Zymography and Immunofluorescent Staining

Frozen sections taken from the limbal explant cultures were mounted on silane-coated slides and incubated with assay kits (EnzChek Gelatinase/Collagenase; Invitrogen, Carlsbad, CA) for the activity of MMP-9 at 37°C in a light-protected, humidified chamber for 20 hours. After incubation, the samples were rinsed twice with PBS to remove nonspecific binding. To block nonspecific staining, the slides were incubated in blocking solution including 1% normal goat serum and 4% BSA for 30 minutes. Sections were incubated with an anti-MMP-9 (NeoMarker; Thermo Fisher Scientific, Rochester, NY) or anti-uPA (Biogenesis, Poole, UK) antibody at a dilution of 1:100 for 1 hour at room temperature. After interaction, the sections were washed with PBS and then incubated with rhodamine-conjugated secondary antibody for MMP-9 and AMCA-conjugated antibody for uPA, respectively, at a dilution of 1:100 for 1 hour at room temperature. The slides were then washed and scanned using a confocal microscope (Leica Confocal Laser Scanning Microscopy; Leica, Deerfield, IL). Gelatinase activates the quenched fluorescent substrate, producing areas of fluorescence against a dark background.

Preparation of Cell Extracts and Western Immunoblot Analysis

Expanded epithelial sheets on AM were washed and scraped off with a spatula. Cell lysates were prepared as previously described.[23] Human Ln5 γ2-chain native protein was purchased from Abcam (Cambrige, MA). To investigate the effects of MMP-9 and uPA on processing of Ln5 γ2-chain, the respective neutralizing antibodies were used to remove the MMP-9 and uPA molecules from cultured media saved from the H/A^{intact} group at day 21. Then, Ln 5 γ2-chain was incubated with the day 21 cultured media and the fractions of supernatant after immunoprecipitation at 37°C for 24 hours. After incubation, total protein (25 μg) from each sample was subjected to SDS–PAGE using a 12% running gel, transferred to nitrocellulose membrane, and then incubated with an anti-uPA (Biogenesis), anti-GAPDH (Biogenesis) or anti-Ln5 γ2-chain (DakoCytomation, Copenhagen, Denmark) antibodies used at a dilution of 1:1000 in TTBS overnight at 4°C. Membranes were washed with TTBS four times for 5 minutes each, incubated with a 1:2000 dilution of anti-rabbit and anti-mouse horseradish peroxidase antibodies for 1 hour, respectively. The membranes were washed with TTBS. The immunoreactive bands detected by ECL reagents were developed by Hyperfilm-ECL.

RT-PCR and Real-Time Quantitative-PCR Analysis

Total RNA was isolated from human limbal epithelial cells cultured on intact AM, plastic dish or AM alone, with Trizol (Invitrogen) according to the manufacturer's instructions. PCR was performed with the following primers (100 ng/μL concentration) for uPA (sense, 5′-GACTCCAAAGGCAGCAATG-3′; anti-sense, 5′-CGATGGTGGTGAATTCTCC-3′); MMP-9 (sense, 5′-GGCGCTCATGTACCCTATGT-3′; antisense, 5′-TCAAAGACCGAGTCCAGCTT-3′). The amplification of β-actin (sense, 5′-GACGGGGTCACCCACACTGTGCCCATCTA-3′; anti-sense, 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′) was used to verify that equal amounts of RNA were loaded for RT-PCR amplification from different experimental conditions. The annealing temperature was 58°C for uPA and 55°C for β-actin. Amplified fragment sizes for uPA, MMP-9, and β-actin were 514 bp, 468 bp, and 636 bp, respectively. The resulting PCR product was analyzed by 2% agarose gel electrophoresis.

Furthermore, using the cDNA templates, real-time quantitative PCR (QPCR) was performed according to the manufacturer's protocols (Applied Biosystems, Foster City, CA). Assays were performed using a real-time PCR system using a mix (StepOnePlus with TaqMan Universal PCR Master Mix; Applied Biosystems; http://www.appliedbiosystems.com). The uPA (Hs01547054_m1), MMP-9 (Hs00957555_m1), GAPDH (Hs99999905_m1) primers and probe were designed using commercial software (ABI PRISM Sequence Detection System; Applied

Biosystems). The probes were labeled with a FAM dye. The data are calculated with ΔΔCt. All quantitative PCR assays were performed in triplicates. Results were expressed as ratios of target gene mRNA copies to GAPDH copies.

Statistical Analysis

Quantitative data were analyzed by a computer program (Prism 4; GraphPad, San Diego, CA), expressed as the mean ± SE, and analyzed with a two-tailed Student's *t*-test with $P < 0.05$ set as the level of significance between individual groups.

Results

Expression of uPA in Human Limbal Epithelial Cells Cultivated on Intact AM

To determine whether uPA was induced by limbal epithelial cells and correlated the temporal relationship to MMP-9 expression, the culture media were collected from three culture conditions: human limbal explants cultured on intact AM (H/A^{intact} group); on plastic dishes (H group); and intact AM (AM group) alone (i.e., without explants); and assessed by fibrin or gelatin zymographic analyses. As shown in Figure 1A, the expression of uPA was upregulated in both H/A^{intact} and H groups. The levels of uPA in H/A^{intact} and H groups at the third week in culture were approximately 4.8 ± 0.55- and 3.7 ± 0.39-fold of those at the first week, respectively. However, no activity of uPA was detected in the AM group. The temporal expression pattern of MMP-9 in limbal explant cultures was similar to that of uPA, demonstrating that active form of MMP-9 in H/A^{intact} group, but not in H and A groups, was upregulated in a time-dependent manner (Fig. 1B).

uPA Protein and mRNA Expression in Limbal Explants Cultivated on Intact AM

To clarify whether the protein levels of uPA were upregulated and correlated with its activity, the proteins were harvested to compare the expression of uPA between H/A^{intact} and H groups. As shown in Figure 2A, the uPA protein expression was significantly increased in both groups in a time-dependent manner, the levels of which at the third week were approximately 2.1 ± 0.2- and 1.5 ± 0.08-fold of those in the first week, respectively ($P < 0.01$, $n = 8$). Furthermore, the protein level of uPA at the third week in H/A^{intact} group was significantly higher than that in the H groups ($P < 0.05$, $n = 8$), suggesting that uPA proteins were preferentially secreted in limbal explant cultured on intact AM. We next analyzed uPA expression at the transcriptional level. As demonstrated in Figure 2B, the uPA transcripts were higher in the H/A^{intact} group than those in the H groups ($P < 0.01$, $n = 4$) by RT-PCR (upper panel) and real-time QPCR (lower panel). These results suggested that uPA mRNA transcripts, protein expression, and enzymatic activity were significantly upregulated in H/A^{intact} group.

Co-localization of MMP-9 and uPA Activities on Intact AM-Expanded Limbal Epithelial Cells

It has been demonstrated that the activities of MMP-9 and uPA at the leading edges were associated with corneal epithelial migration.[24,25] We therefore investigated whether the activity of MMP-9 was co-localized with uPA by immunofluorescent staining. As shown in Figure 3, in situ MMP-9 activity (A) and the distribution of uPA (B) were well co-localized within the leading edge of limbal epithelial cells on intact AM (C). These data revealed a causal relationship between uPA and MMP-9 expression in modulating limbal outgrowth in this model.

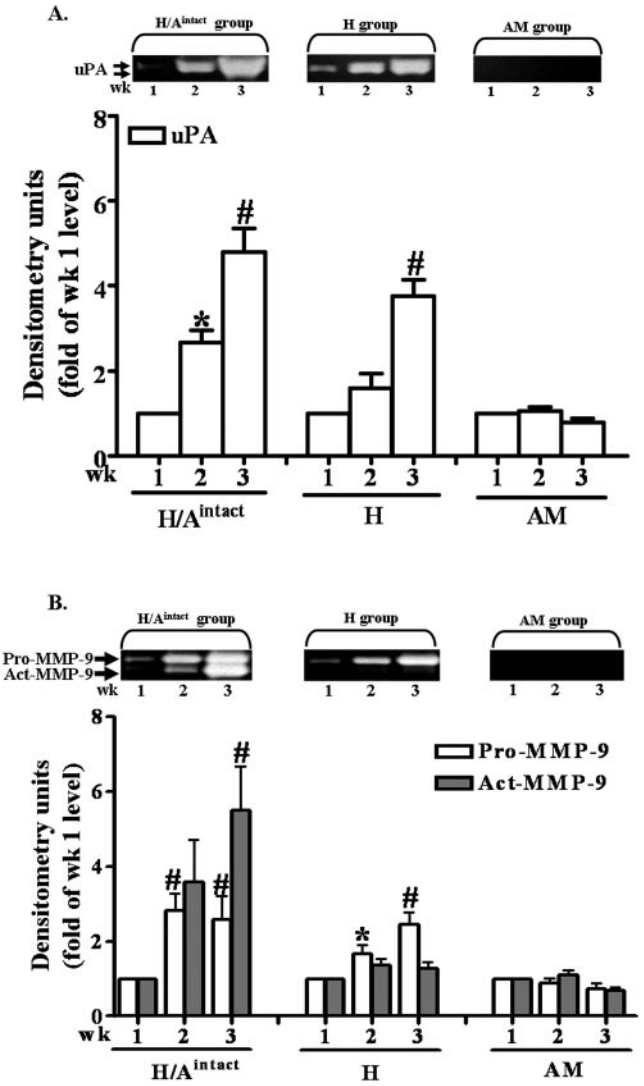

Figure 1. The expression of uPA and MMP-9 in limbal explant cultures on intact AM (H/A^{intact} group), plastic dish (H group), and intact AM (AM group) alone (i.e., without explant) for the indicated time. (**A**) uPA and (**B**) MMP-9 levels in various culture conditions were determined and quantified by fibrin and gelatin zymography, respectively. The media were collected from H/A^{intact} group, H group, and AM group, at indicated time. Equal amounts of proteins were loaded and the expressions of uPA and MMP-9 were analyzed by zymography and quantified by a densitometer of the corresponding bands in the linear response range of the zymography. The zymographs shown represented one of at least six individual experiments. $^{*}P < 0.05$; $^{\#}P < 0.01$, compared with the respective levels of pro–MMP-9 and uPA expression at the first week in H/A^{intact} and H groups. $^{\#}P < 0.01$, compared with active-MMP-9 expression at the first week in H/A^{intact} group.

Effect of uPA Inhibitor, B428, on MMP-9 Activity and mRNA Expression

Pretreatment with B428, a selective uPA inhibitor, at the end of second week apparently retarded the active form MMP-9 compared with the control, determined by gelatin zymography (Fig. 4A), implying that uPA was upstream of MMP-9 and capable of regulating its activation. To verify whether MMP-9 was transcriptionally regulated by uPA, total RNA was harvested among various conditions including H/A^{intact} treated without or with B428 (20 μM), H, and AM groups. As demonstrated in Figure 4B, the MMP-9 mRNA expression was higher in H/A^{intact} group than all the other conditions by RT-PCR

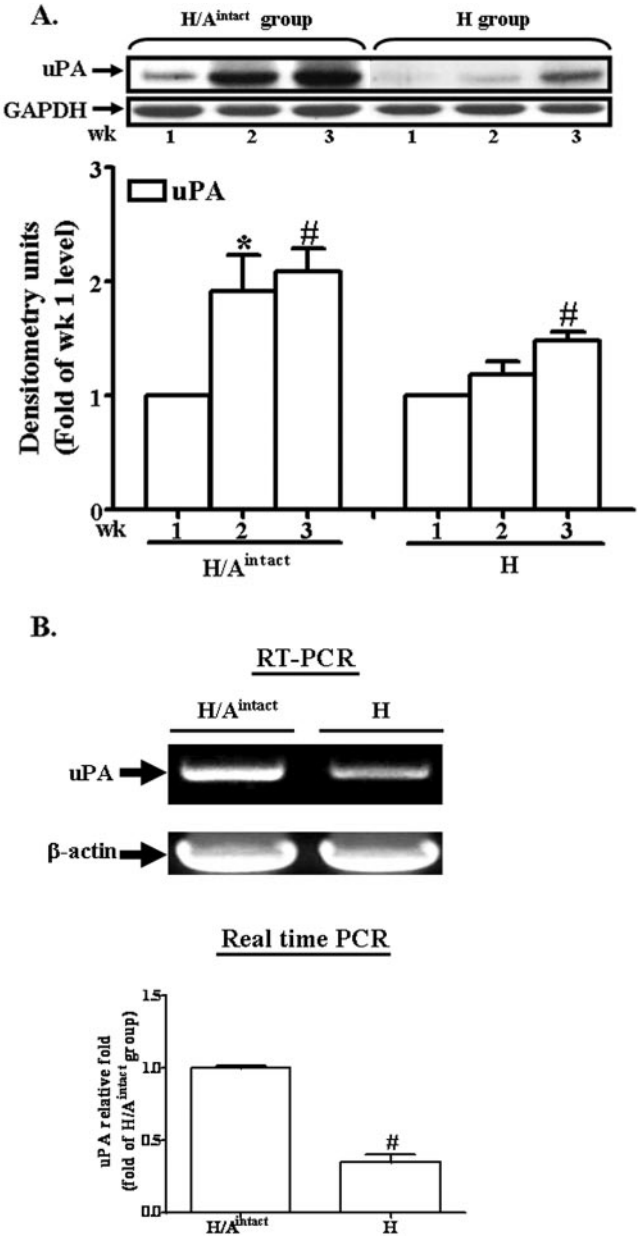

FIGURE 2. Time-dependent overexpression of uPA protein and mRNA in H/A^{intact} group versus in H group at indicated time. (**A**) The expressions of uPA proteins in various culture conditions were determined and quantified by Western blot. The proteins were collected from H/A^{intact} group and H group at indicated time. Equal amounts of proteins were subjected to 10% SDS-PAGE and analyzed by Western blotting. Bands were visualized by an ECL method and quantified by a densitometer. The bands shown represent one of at least six individual experiments. $^{*}P < 0.05$; $^{\#}P < 0.01$. (**B**) Differential expression of uPA mRNA of H/A^{intact} group and H group. After culture for 3 weeks, the total RNA was extracted and analyzed by RT-PCR and real-time quantitative-PCR. The N-fold differential expression of uPA mRNA in H group related to H/A^{intact} group was determined and expressed by $2^{-\Delta\Delta Ct}$. β-actin and GAPDH served as endogenous control. Data are summarized and expressed as the mean ± SE of six individual experiments. $^{\#}P < 0.01$, compared with H/A^{intact} group.

(upper panel) and real-time QPCR (lower panel). The expression of MMP-9 in B428-treated H/A^{intact} group and H group normalized to H/A^{intact} control were 0.469 ± 0.165 and 0.018 ± 0.004, respectively. There was hardly any RNA isolated from AM alone, consistent with our previous study.[22] These results indicated that uPA regulated MMP-9 expression at transcriptional level in this model.

uPA/Plasmin Regulating MMP-9 Expression in Limbal Epithelial Cells Expanded on Intact AM

Since uPA participates in the conversion of plasminogen to plasmin, we hypothesized that PA/plasmin system was involved in the activation of MMP-9 by uPA. To test this possibility, the conditioned media at day 17, 19, and 21 from H/A^{intact} and H groups were collected, pre-incubated with plasminogen and subjected to fibrin zymographic analysis. As shown in Figure 5A, the level of plasmin expression was higher in H/A^{intact} group than that in H group, consistent with the finding that uPA activity was preferentially expressed in H/A^{intact} group (Fig. 1A). To investigate whether plasmin was involved in activating MMP-9 expression, the collected condition media from H/A^{intact} and H groups were pre-incubated with plasmin and subjected to gelatin zymographic analysis. Results in Figure 5B showed that the expression of active form MMP-9 was enhanced in H/A^{intact} group. In contrast, pretreatment with plasmin had little effect on the production of active form MMP-9 in H group, indicating that plasmin induced active form MMP-9 only when human limbal epithelial cells were expanded on intact AM. As shown in Figure 5C (lower panel), the active form MMP-9 was increased when plasmin was added to culture medium compared with the control. These results implied that plasmin was involved in MMP-9 activation in limbal epithelial cells expanded on intact AM. To confirm the expression of MMP-9 induced by plasmin was mediated through uPA activation, condition media collected from H/A^{intact} group treated with B428 were subjected to fibrin zymography. As shown in Figure 5C (upper panel), the production of plasmin was inhibited by B428 compared with that of the control, indicating that generation of plasmin was regulated by uPA. To verify that MMP-9 activity was regulated by PA/plasmin, limbal epithelial cells on intact AM were treated with B428, sectioned and then stained with in situ gelatin zymography. To co-localize the expression of MMP-9, tissue sections were also incubated with an anti-MMP-9 antibody. As shown in Figure 5D, the activity (in situ zymography) and expression (immunofluorescent staining) of MMP-9 were attenuated (V, VI, and VIII) in cultures treated with B428 compared with the control (I, II, and IV). These results implied that uPA/plasmin participated in MMP-9 activation in limbal epithelial cells cultivated on intact AM, rather than on plastic dishes.

uPA/Plasmin/MMP-9 Axis–Involved Processing of Ln5 γ2-Chain and Outgrowth of Limbal Explants Cultured on Intact AM

Ln5 is an ECM component of amniotic basement membrane involved in cell adhesion and migration.[16] Proteolytic processing of the Ln5 γ2-chain by MT1-MMP and MMP-2 may result in release of EGF-like repeats[20,21], leading to epithelial cell migration.[26,27] To identify whether the Ln5 γ2-chain was proteolytically degraded in this model, we harvested proteins from conditioned media to compare the expression of fragments of human Ln5 γ2-chain between H/A^{intact} and AM groups by Western blot analysis. As demonstrated in Figures 6A and 6B, the 155 kDa Ln5 γ2-chain of human AM was preferentially processed into ~50, ~42, and ~36 kDa novel fragments in H/A^{intact} group, but not in AM group. The expression pattern of ~50 kDa novel fragment of human Ln5 γ2-chain was similar to those of MMP-9 and uPA in a time-dependent manner (Fig. 6A). Moreover, treatment of limbal cultures with 10 μM GM6001 or 0.3 μM MMP-2/-9 inhibitor for one week attenuated the expression of ~50 kDa fragment of human Ln5 γ2-chain

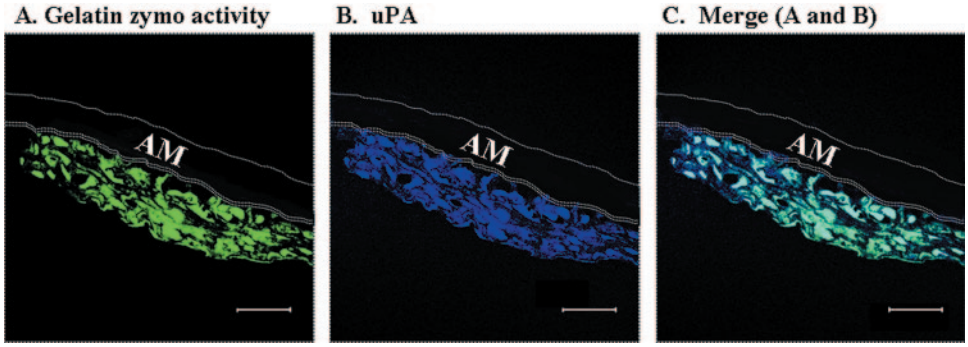

FIGURE 3. Co-localization of MMP-9 and uPA activities at the leading edge of ex vivo expanded human limbal epithelial cells on intact AM. The activities of MMP-9 and uPA were determined by in situ gelatin zymographic analysis and immunofluorescent staining, respectively. MMP-9 enzymatic activity (**A**) and uPA expression (**B**) were well co-localized within the region of migration front (**C**) of intact AM-expanded limbal epithelial cells. *Green*: in situ gelatin zymographic activity. *Blue*: uPA expression. The merged picture of (**A**) and (**B**) was unrolled in (**C**). Scale bars, 40 μm. Magnification, $\times$630.

compared with control (Fig. 6C), indicating that the production of ~50 kDa fragment was mediated through proteolysis of human Ln5 γ2-chain by MMP-9. Also, the generation of ~50 kDa fragment of human Ln5 γ2-chain was inhibited by treatment with B428 in H/A^{intact} culture (Fig. 6C), proving that uPA is capable of regulating MMP-9 activity. To further confirm that Ln5 γ2-chain is digested by uPA and MMP-9, the purified Ln5 γ2-chain protein was incubated with conditioned media collected from the groups tested. As shown in Figure 6D, the intact Ln5 was cleaved into a ~50 kDa novel fragment in normal cultured media. The processing of Ln5 was significantly attenuated by the cultured media pretreated with respective neutralizing antibody MMP-9 or uPA compared with the basal group. These results directly demonstrated that the cleavage of Ln5 γ2-chain is mediated through MMP-9 and uPA in this response. Activation of uPA and MMP-9 has been implicated in epithelial cell migration and proliferation.[22,25] To verify whether intact AM-expanded limbal outgrowth was regulated by PA/plasmin, cultures of limbal epithelial outgrowth were treated with or without B428 (20 μM) at the end of the second week. One week later, treatment with B428 evidently retarded limbal epithelial outgrowth compared with the control (Fig. 6E). The corrected ratio of outgrowth area of limbal epithelial cells in B428-treated cultures normalized to that of control in H/A^{intact} group was 0.49 $\pm$ 0.06 ($P < 0.01$, $n = 7$). These data suggested that PA/plasmin/MMP-9 axis mediated outgrowth of limbal epithelial cells on intact AM through processing of Ln5 γ2-chain.

DISCUSSION

uPA, together with MMP-9, are normal components of the tear fluid originating from conjunctival and corneal epithelial cells and corneal endothelium.[28,29] We have demonstrated that the expression of MMP-9, facilitating outgrowth of limbal epithelial cells, is upregulated in limbal explant culture on intact AM[22] However, the mechanisms regulating the activity and expression of MMP-9 in this model were not completely clarified. In this study, we have shown that uPA activity, similar to that of MMP-9, was upregulated during the outgrowth of limbal epithelial cells on intact AM. We further verified the spatial relationship between uPA and MMP-9 by co-localizing their activities at the leading edge of expanded limbal epithelial cells. Moreover, the expression of uPA was also upregulated and paralleled with MMP-9 expression in the H/A^{intact} group at both the transcriptional and translational levels. Finally, we identified that the activity of MMP-9 was regulated by the

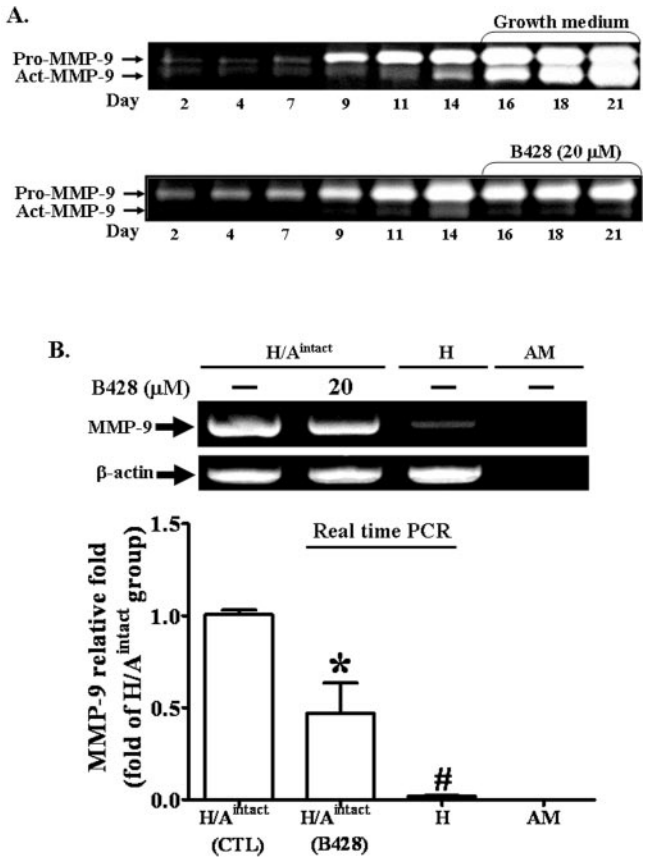

FIGURE 4. Treatment with a uPA inhibitor, B428, during day 16 to 21 attenuated the activity and expression of MMP-9. (**A**) Condition media collected from H/A^{intact} group treated without or with B428 (20 μM) were analyzed by gelatin zymography. (**B**) Differential expression of MMP-9 mRNA among cultures of H/A^{intact} with or without B428 (20 μM), H, and AM groups. After incubation for 3 weeks, the total RNA was extracted and analyzed by RT-PCR and real-time quantitative-PCR. Intact AM-expanded limbal cultures treated with B428 downregulated MMP-9 mRNA transcripts compared with the control. The N-fold differential expression of MMP-9 mRNA in various groups related to H/A^{intact} group was determined and expressed by $2^{-\Delta\Delta Ct}$. β-actin and GAPDH served as internal standard. $^{*}P < 0.05$ compared with H/A^{intact} group without B428 treatment; $^{\#}P < 0.01$, compared with H/A^{intact} group. The real-time quantitative-PCR shown represented one of at least six individual experiments.

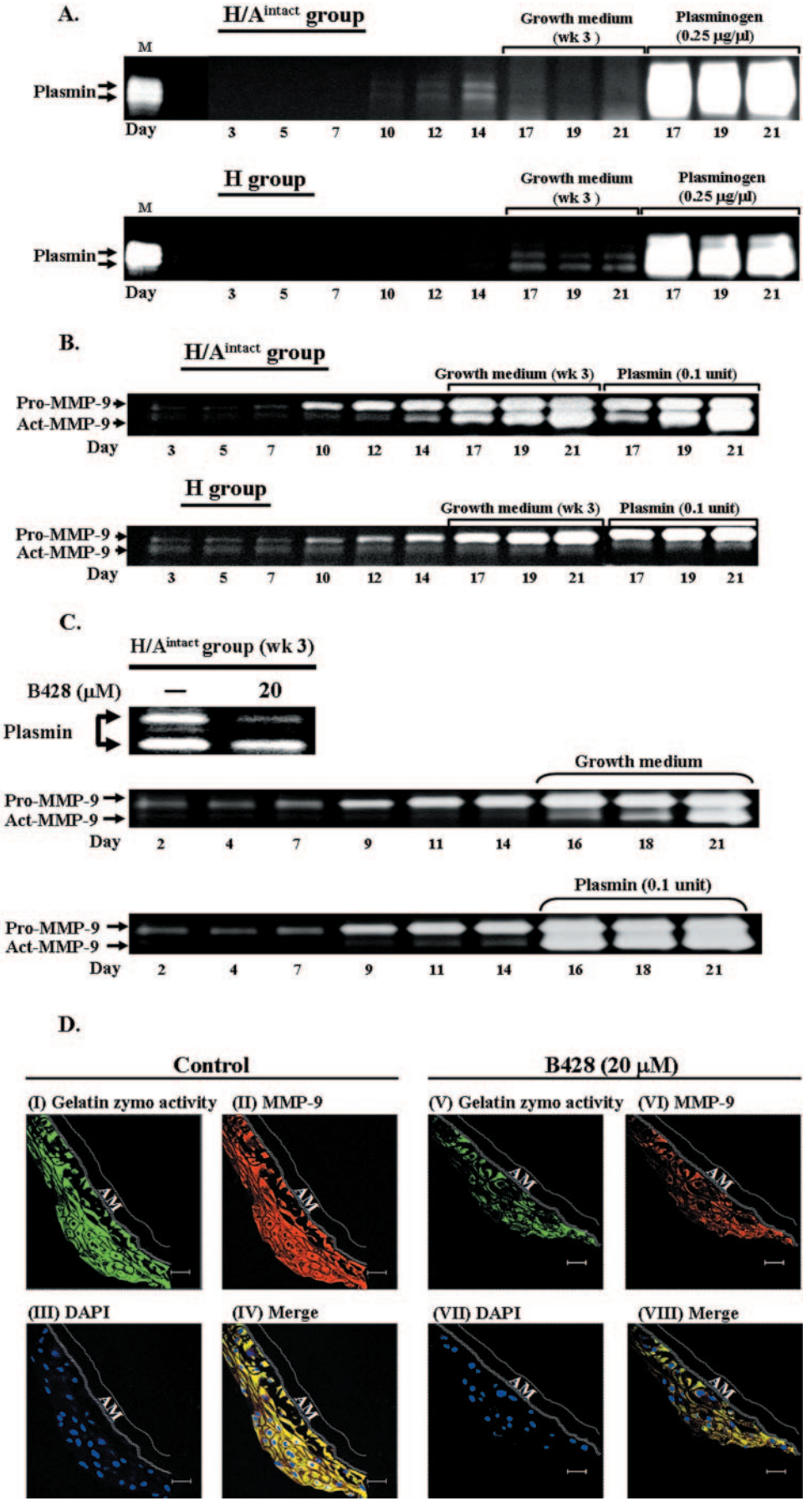

FIGURE 5. Plasmin regulated active form MMP-9 expression in intact AM-expanded human limbal epithelial cells. (**A**) The culture supernatants at day 17, day 19, and day 21 from both H/A^{intact} and H groups were harvested and pre-incubated with 0.25 μg/mL plasminogen and subjected to fibrin zymographic analysis. (**B**) The supernatants at day 17, day 19, and day 21 from both H/A^{intact} and H groups were harvested and pre-incubated with 0.1 unit plasmin and subjected to gelatin zymographic analysis. (**C**) Condition media collected from H/A^{intact} group treated with or without 0.1 unit plasmin and 20 μM B428 at day 14 for 1 week were subjected to gelatin and fibrin zymographic analysis, respectively. The zymographs shown represented one of at least six individual experiments. M, molecular marker of plasmin. (**D**) Intact AM-expanded limbal epithelial cells treated without or with B428 were frozen-sectioned at the end of the 3rd week. To co-localize the activity and expression of MMP-9, tissue sections were incubated with gelatin substrate overnight, followed by staining with an MMP-9 antibody and photographed by a confocal microscope. The activity and expression of MMP-9 were attenuated (V, VI, and VIII) in cultures treated with B428 compared with the control (I, II, and IV). *Green*: in situ gelatin zymographic activity. *Red*: MMP-9 expression. *Blue*: DAPI for nuclear staining. Figures (IV) and (VIII) were the merged pictures of (I, II, and III) and (V, VI, and VII). Scale bars, 40 μm. Magnification, $\times$400.

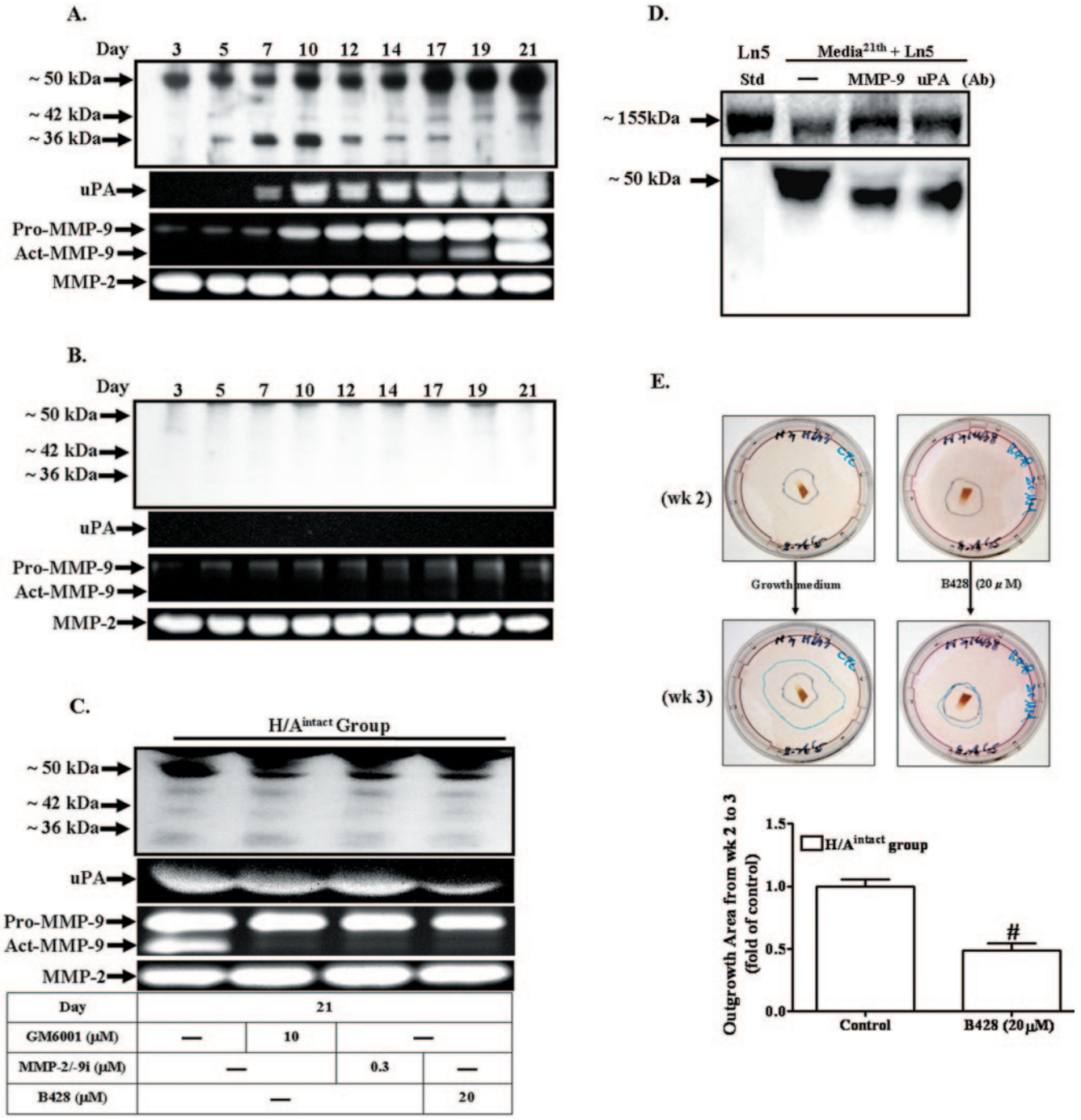

FIGURE 6. uPA/plasmin/MMP-9 axis involved in the processing of Ln5 γ2-chain and outgrowth of limbal explants cultured on intact AM. The media were collected from the (**A**) H/A^{intact}, (**B**) AM groups at the indicated time, and (**C**) Sham-, GM6001 (10 μM)–, MMP-2/-9 inhibitor (0.3 μM)–, and B428 (20 μM)–treated H/A^{intact} groups at day 21. (**D**) Human Ln5 γ2-chain protein after incubation with conditioned media collected from of H/A^{intact} group at day 21 and supernatant fractions of immunoprecipitation (MMP-9 and uPA antibodies, respectively) followed by Western blotting. Equal amounts of proteins were resolved by 12% SDS-PAGE and analyzed by Western blotting, using an anti-human Ln5 γ2-chain monoclonal antibody. Bands were visualized by ECL. The uPA and MMP-9 activities were determined by fibrin and gelatin zymography, respectively. Intact Ln5 γ2-chain was present in the ~155 kDa form. The expression of fragments of human Ln5 γ2-chain, uPA, and MMP-9 were different between the H/A^{intact} and AM groups for the indicated periods. The processing of intact Ln5 γ2-chain into a ~50 kDa novel fragment was decreased after uPA or MMP-9 immunoprecipitation. The bands and zymographs shown are representative of one of at least six individual experiments with similar results. (**E**) The same sizes of limbal epithelial outgrowth at the end of the 2nd week were selected and treated with or without B428 (20 μM) for 1 week. After 1 week of treatment, the area of limbal epithelial outgrowth was decreased compared with the control. The figures shown were representative of at least seven individual experiments. Data are summarized and expressed as the mean ± SE of seven independent experiments. $^{\#}P < 0.01$, compared with control. std, standard.

uPA/plasmin, which mediated proteolytic processing of Ln5 γ2-chain and regulated limbal epithelial outgrowth on intact AM (Fig. 7).

MMPs and uPA are secreted by cells as inactive pro-enzymes and must bind to the cell surface, where they are processed and activated.[12,30] Cell surface activation of MMP-2 can occur

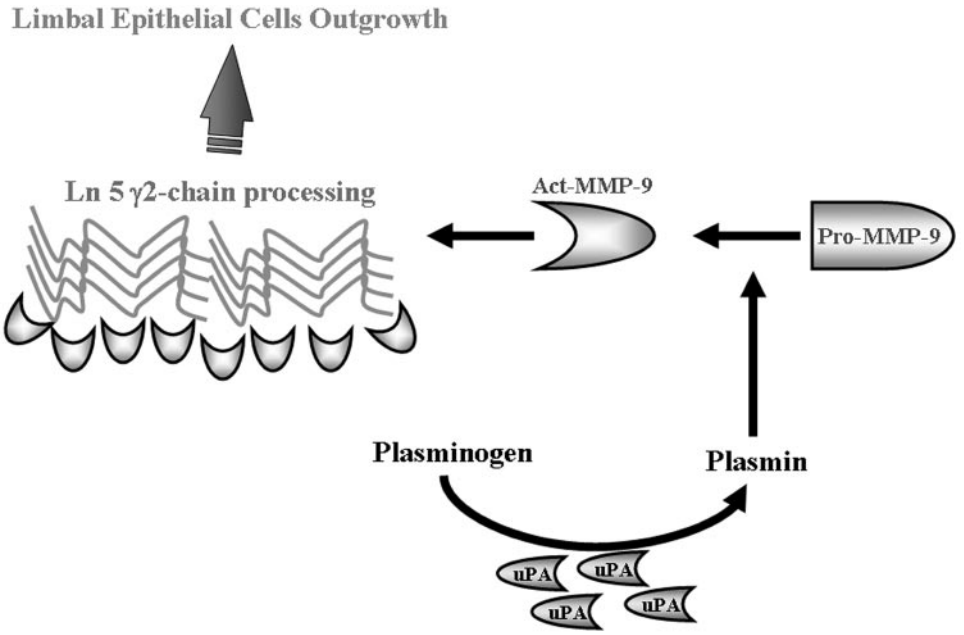

FIGURE 7. Schematic pathways of uPA/Plasmin axis regulate MMP-9 activity and Ln5 γ2-chain degradation on intact AM-expanded limbal epithelial cells. Active uPA converts plasminogen to plasmin at the surface of limbal epithelial cells. Plasmin activates pro–MMP-9 associated with leading to Ln5 γ2-chain processing and promotes limbal epithelial outgrowth on intact AM.

when it is brought into contact with a membrane-associated MMP, MT1-MMP.[30] Interestingly, MMP-9, a close structural homologue of MMP-2, is not activated by the same mechanism.[31] Other proteases, such as plasmin,[14,15] MMP-7,[32] MMP-3,[33] and MMP-13[34] have been reported to activate pro–MMP-9 in vitro. Plasmin was generated from plasminogen by the endogenous uPA.[12] In situ synthesis of intact plasminogen molecule (87 kDa) has been found in all three layers of human cornea.[35] Moreover, mechanical wound has been shown to induce uPA expression in corneal epithelial cells,[25] implying a critical role of uPA/plasmin during corneal epithelial wound healing and migration. Overexpression of uPA in H/A^{intact} group (Figs. 1, 2) indicated that uPA is possibly involved in ECM degradation during outgrowth of limbal epithelial cells on intact AM.

The expression of MMP-9 has been identified in lamellipodia of migrating cells close to the cell-ECM junctions.[36] It has also been demonstrated that uPA is present at the leading edge of the migrating corneal epithelium.[25] The fact that uPA and MMP-9 activities were co-localized at the leading edge of expanded limbal epithelial cells (Fig. 3) implied their concerted efforts in ECM processing. We further demonstrated a critical role of uPA in mediating MMP-9 expression by showing that B428 attenuated the activity of MMP-9 and mRNA transcripts in expanded limbal epithelial cells (Fig. 4). The activity of uPA was not changed by treatment with GM6001 (data not shown), eliminating the possibility that MMP-9 might modulate uPA activity and expression. Collectively, these results suggested that uPA was an upstream component of MMP-9 and regulated the activity of MMP-9 in this model.

A number of studies have recognized plasmin as a direct activator of pro–MMP-9,[14] although there are contradictory reports.[37] However, the role of plasmin in activating MMP-9 in this model was not clear. By supplementation of exogenous plasminogen, plasmin was expressed in both H/A^{intact} and H groups (Fig. 5A). The higher level of plasmin in H/A^{intact} group, consistent with that of uPA activity (Fig. 1A), may indirectly reflect that most of the plasminogen was converted to plasmin by uPA. In addition, we found a temporal relationship between plasmin expression and MMP-9 activation in H/A^{intact} group. Therefore, it seems very plausible to hypothesize that in the presence of intact AM, the high level of endogenous plasmin produced by uPA initiates MMP-9 activation. To confirm this finding, we demonstrated the capability of plasmin on activating pro–MMP-9 by showing that exogenous plasmin upregulated active form MMP-9 in HA^{intact} group (Fig. 5B). The result was similar with the pattern of MMP-9 expression as in our previous study,[22] and implied that the presence of intact AM was necessary for the induction of the active form MMP-9. Similar finding was observed by adding plasmin into HA^{intact} group at the end of the second week in culture (Fig. 5C). Finally, attenuated plasmin production by B428 in intact AM expanded-limbal epithelial cells further confirmed that plasmin-regulated MMP-9 activation was mediated through the activation of uPA. These results indicated that uPA/plasmin was a direct activator of pro–MMP-9 in limbal epithelial cells expanded on intact AM. However, further study is mandatory to investigate other possible molecules, such as MMP-3, participating in the regulation of MMP-9 activity.

MMP-2 and MMP-9 have been reported to be involved in basement membrane dissolution and reassembly in the same culture model.[38] Epithelial outgrowth from the explant correlated with a dramatic increase of MMP-9 in the conditioned medium of intact AM cultures. They also note that Ln5 is dissolved about one week and re-deposited from week 3 to 4 in explant culture on intact AM. In epidermal keratinocytes, absence of Ln5 triggers activation of p38 MAPK[39] and up-regulates MMP-9 expression,[40] leading to corneal epithelial migration.[41] These studies reveal that ECM degradation, especially the Ln5, plays a key role in ex vivo limbal culture on AM. In the present study, novel proteolytic fragments of Ln5 γ2-chain consisting of ~50, ~42, and ~36 kDa were found in H/A^{intact} group rather than in AM group (Figs. 6A, 6B). Interestingly, the expression of the ~50 kDa protein was temporally paralleled with and regulated by the activities of uPA and MMP-9 (Figs. 6A, 6C, 6D). It is also noteworthy that the ~42 kDa novel proteolytic fragment was well correlated to activation of MMP-9. Although the respective functions of these novel fragments were not clarified, our data were consistent with the study showing that basement membrane remodeling is necessary for limbal explants cultured on intact AM[38]

In summary, our data suggested a strong correlation between uPA expression and MMP-9 activity, and the activity of which was necessary at the migrating front to carve out a path for facilitating subsequent epithelial proliferation through ECM remodeling such as Ln5 γ2-chain in this culture model of human limbal explants on intact AM. However, further studies

are still necessary to investigate the characteristics and significance of the novel fragments of Ln5 γ2-chain and to explore the associated extra- and intra-cellular signaling events in the future.

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References

1. Lee SH, Tseng SC. Amniotic membrane transplantation for persistent epithelial defects with ulceration. *Am J Ophthalmol.* 1997; 123:303–312.
2. Kruse FE, Rohrschneider K, Völcker HE. Multilayer amniotic membrane transplantation for reconstruction of deep corneal ulcers. *Ophthalmology.* 1999;106:1504–1510.
3. Gomes JA, dos Santos MS, Cunha MC, et al. Amniotic membrane transplantation for partial and total limbal stem cell deficiency secondary to chemical burn. *Ophthalmology.* 2003;110:466–473.
4. Tsai RJF, Li LM, Chen JK. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. *N Engl J Med.* 2000;343:86–93.
5. Koizumi N, Inatomi T, Suzuki T, et al. Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. *Ophthalmology.* 2001;108:1569–1574.
6. Dua HS, Gomes JA, King AJ, et al. The amniotic membrane in ophthalmology. *Surv Ophthalmol.* 2004;49:51–77.
7. Sun CC, Su Pang JH, Cheng CY, et al. Interleukin-1 receptor antagonist (IL-1RA) prevents apoptosis in ex vivo expansion of human limbal epithelial cells cultivated on human amniotic membrane. *Stem Cells.* 2006;24:2130–2139.
8. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res.* 2003;92:827–839.
9. Sidenius N, Blasi F. The urokinase plasminogen activator system in cancer: recent advances and implication for prognosis and therapy. *Cancer Metastasis Rev.* 2003;22:205–222.
10. Blasi F, Carmeliet P. uPAR: a versatile signalling orchestrator. *Nat Rev Mol Cell Biol.* 2002;3:932–943.
11. Collen D, Lijnen HR. Thrombolytic agents. *Thromb Haemost.* 2005;93:627–630.
12. Petersen LC, Lund LR, Nielsen LS, et al. One-chain urokinase-type plasminogen activator from human sarcoma cells is a proenzyme with little or no intrinsic activity. *J Biol Chem.* 1988;263:11189–11195.
13. Desrivieres S, Lu H, Peyri N, et al. Activation of the 92 kDa type IV collagenase by tissue kallikrein. *J Cell Physiol.* 1993;157:587–593.
14. Saunders WB, Bayless KJ, Davis GE. MMP-1 activation by serine proteases and MMP-10 induces human capillary tubular network collapse and regression in 3D collagen matrices. *J Cell Sci.* 2005; 118:2325–2340.
15. Fu X, Parks WC, Heinecke JW. Activation and silencing of matrix metalloproteinases. *Semin Cell Dev Biol.* 2008;19:2–13.
16. Kurpakus-Wheater M. Laminin-5 is a component of preserved amniotic membrane. *Curr Eye Res.* 2001;22:353–357.
17. Ogura Y, Matsunaga Y, Nishiyama T, et al. Plasmin induces degradation and dysfunction of laminin 332 (laminin 5) and impaired assembly of basement membrane at the dermal-epidermal junction. *Br J Dermatol.* 2008;159:49–60.
18. Carter WG, Ryan MC, Gahr PJ. Epiligrin, a new cell adhesion ligand for integrin alpha 3 beta 1 in epithelial basement membranes. *Cell.* 1991;65:599–610.
19. Baker SE, Hopkinson SB, Fitchmun M, et al. Laminin-5 and hemidesmosomes: role of the alpha 3 chain subunit in hemidesmosome stability and assembly. *J Cell Sci.* 1996;109:2509–2520.
20. Giannelli G, Falk-Marzillier J, Schiraldi O, et al. Induction of cell migration by matrix metalloprotease-2 cleavage of laminin-5. *Science.* 1997;277:225–228.
21. Koshikawa N, Giannelli G, Cirulli V, et al. Role of cell surface metalloprotease MT1-MMP in epithelial cell migration over laminin-5. *J Cell Biol.* 2000;148:615–624.
22. Sun CC, Cheng CY, Chien CS, et al. Role of matrix metalloproteinase-9 in ex vivo expansion of human limbal epithelial cells cultured on human amniotic membrane. *Invest Ophthalmol Vis Sci.* 2005; 46:808–815.
23. He H, Cho HT, Li W, et al. Signaling-transduction pathways required for ex vivo expansion of human limbal explants on intact amniotic membrane. *Invest Ophthalmol Vis Sci.* 2006;47:151–157.
24. Mohan R, Chintala SK, Jung JC, et al. Matrix metalloproteinase gelatinase B (MMP-9) coordinates and effects epithelial regeneration. *J Biol Chem.* 2002;277:2065–2072.
25. Watanabe M, Yano W, Kondo S, et al. Up-regulation of urokinase-type plasminogen activator in corneal epithelial cells induced by wounding. *Invest Ophthalmol Vis Sci.* 2003;44:3332–3338.
26. Hintermann E, Quaranta V. Epithelial cell motility on laminin-5: regulation by matrix assembly, proteolysis, integrins and erbB receptors. *Matrix Biol.* 2004;23:75–85.
27. Pirila E, Sharabi A, Salo T, et al. Matrix metalloproteinases process the laminin-5 gamma 2-chain and regulate epithelial cell migration. *Biochem Biophys Res Commun.* 2003;303:1012–1017.
28. Fini ME, Girard MT. Expression of collagenolytic/gelatinolytic metalloproteinases by normal cornea. *Invest Ophthalmol Vis Sci.* 1990;31:1779–1788.
29. Tripathi RC, Tripathi BJ, Park JK. Localization of urokinase-type plasminogen activator in human eyes: an immunocytochemical study. *Exp Eye Res.* 1990;51:545–552.
30. Murphy G, Stanton H, Cowell S, et al. Mechanisms for pro matrix metalloproteinase activation. *APMIS.* 1999;107:38–44.
31. Ward RV, Atkinson SJ, Slocombe PM, et al. Tissue inhibitor of metalloproteinases-2 inhibits the activation of 72 kDa progelatinase by fibroblast membranes. *Biochim Biophys Acta.* 1991;1079: 242–246.
32. von B, Cress AE, Howard EW, et al. Activation of gelatinase-tissue-inhibitors-of-metalloproteinase complexes by matrilysin. *Biochem J.* 1998;331:965–972.
33. Ogata Y, Enghild JJ, Nagase H. Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. *J Biol Chem.* 1992;267:3581–3584.
34. Han YP, Yan C, Zhou L, et al. A matrix metalloproteinase-9 activation cascade by hepatic stellate cells in trans-differentiation in the three-dimensional extracellular matrix. *J Biol Chem.* 2007;282: 12928–12939.
35. Twining SS, Wilson PM, Ngamkitidechakul C. Extrahepatic synthesis of plasminogen in the human cornea is up-regulated by interleukins-1alpha and -1beta. *Biochem J.* 1999;339:705–712.
36. Legrand C, Gilles C, Zahm JM, et al. Airway epithelial cell migration dynamics. MMP-9 role in cell-extracellular matrix remodeling. *J Cell Biol.* 1999;146:517–529.
37. Okada Y, Gonoji Y, Naka K, et al. Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) from HT 1080 human fibrosarcoma cells. Purification and activation of the precursor and enzymic properties. *J Biol Chem.* 1992;267:21712–21719.
38. Li W, He H, Kuo CL, et al. Basement membrane dissolution and reassembly by limbal corneal epithelial cells expanded on amniotic membrane. *Invest Ophthalmol Vis Sci.* 2006;47:2381–2389.
39. Harper EG, Alvares SM, Carter WG. Wounding activates p38 map kinase and activation transcription factor 3 in leading keratinocytes. *J Cell Sci.* 2005;118:3471–3485.
40. Suarez-Cuervo C, Merrell MA, Watson L, et al. Breast cancer cells with inhibition of p38alpha have decreased MMP-9 activity and exhibit decreased bone metastasis in mice. *Clin Exp Metastasis.* 2004;21:525–533.
41. Saika S, Okada Y, Miyamoto T, et al. Role of p38 MAP kinase in regulation of cell migration and proliferation in healing corneal epithelium. *Invest Ophthalmol Vis Sci.* 2004;45:100–109.