Journal of Cellular Biochemistry 101:1222–1237 (2007)

Roles of MMP/TIMP in Regulating Matrix Swelling and Cell Migration During Chick Corneal Development

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Abstract Tissue remodeling is central to embryonic development. Here, we used immunohistochemistry, Western blotting, and RT-PCR analysis to investigate the roles of matrix metalloproteinases (MMPs) and the related ''a disintegrin and metalloproteinase'' (ADAM) family proteinases in chick corneal development. While MMP-13 was expressed in developing chick corneas from embryonic day (ED) 5 to ED 10, its inhibitor, tissue inhibitors of metalloproteinase-1 (TIMP-1), was expressed from ED 18 to 2 days post-hatching (P2). Early MMP-13 activity may be associated with degradation of type IX collagen from the primary stroma, which loosens the collagen fibrils and facilitates neural crest (NC) cell migration. The membrane-bound and secreted forms of ADAM10 were both detected throughout corneal development, and active ADAM10 formed a cleavage complex with CD44v6, a CD44 splice variant that is a major cell surface adhesion molecule for hyaluronic acid (HA) and has been implicated in cell migration. Both CD44v6 and its ectodomain cleavage products were detected from ED 5 to ED 14, and a broad-spectrum MMP inhibitor blocked ectodomain cleavage in cultured stromal cells. These findings suggest that ADAM10 mediates CD44v6 cleavage in the developing cornea, facilitating NC cell-derived mesenchymal cell migration. Finally, we identified high levels of active membrane-type 3-MMP (MT3-MMP) in developing corneas at ED 7, ED 14, and ED 18. MT3-MMP takes part in MMP-2 activation and possibly also CD44v6 shedding, suggesting that this pathway may be involved in cell migration. These findings collectively show for the first time that multiple MMPs, ADAMs, and TIMPs appear to functionally interact during corneal development. J. Cell. Biochem. 101: 1222–1237, 2007.

Key words: corneal development; matrix metalloproteinases; MT3-MMP; ADAM10; CD44v6; cell migration; ectodomain cleavage; extracellular matrix

Embryologically, the first stage of corneal development begins after the lens vesicle detaches, when the surface ectoderm starts differentiating into the corneal epithelium. By embryonic day (ED) 5, the corneal epithelium begins to synthesize and basally secrete the ECM components required for formation of the primary corneal stroma, including types I, II, IX, XII, and XIV collagens, fibronectin, tenascin, and other proteoglycans [Hay and Revel, 1969; Hendrix et al., 1982; Svoboda et al., 1988; Doane et al., 1996, 2002; Fitch et al., 1998; Akimoto et al., 2002; Marchant et al., 2002]. Subsequently, neural crest (NC) cells migrate periocularly into the primary stroma (PS) beneath the epithelium, forming a sheet-like monolayer that becomes the corneal endothelium.

Abbreviations used: MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; MT-MMP, membrane-type matrix metalloproteinase; ADAM10, a disintegrin and metalloprotease 10; ECM, extracellular matrix; NC, neural crest; HA, hyaluronic acid; ED, embryonic day.

Man-Il Huh and Young-Mi Lee contributed equally to the results of this work.

Grant sponsor: Kyungpook National University Research Fund (2002); Grant sponsor: Korea Research Foundation; Grant number: KRF-2003-070-C00033; Grant sponsor: NIH; Grant number: R01 EY12651; Grant sponsor: Research to Prevent Blindness; Grant sponsor: Walter G. Ross Foundation.

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Received 29 June 2006; Accepted 27 November 2006

DOI 10.1002/jcb.21246

The second stage of corneal development begins when the PS begins to swell. Endothelial cells secrete hyaluronic acid (HA), a large glycosaminoglycan that is able to absorb and retain water, leading to hydrostatic swelling. HA accumulates in the corneal stroma during invasion of the NC cells, facilitating cell migration [Toole and Trelstad, 1971] and generating hydrostatic pressure on adjacent collagen fibrils. Type IX collagen disappears abruptly from the PS during the swelling process, likely allowing further loosening of the collagen fibrils, along with additional matrix expansion [Fitch et al., 1998]. Facilitated by the migratory space created by these changes, a second invasion of NC into the stroma begins by ED 7, and the acellular PS is replaced with a secondary stroma secreted by the invading cells. The formation of the secondary stroma is essentially complete by ED 14 [Trelstad and Coulombre, 1971], and subsequently the stroma begins to thin, due to dehydration by the functional endothelium [Coulombre and Coulombre, 1964; Siegler and Quantock, 2002]. After ED 14, cell migration ceases, HA disappears, and the stroma condenses into its highly transparent adult form [Coulombre and Coulombre, 1958; Hay and Revel, 1969; Toole and Trelstad, 1971; Bard and Hay, 1975; Hay, 1979; Akimoto et al., 2002; Marchant et al., 2002; Siegler and Quantock, 2002; Young et al., 2002].

A large body of evidence indicates that members of the matrix metalloproteinase (MMP) family of enzymes mediate cell migration and extracellular matrix (ECM) proteolysis and remodeling during development. The MMPs constitute a multigene family of over 25 secreted and cell surface enzymes capable of processing or degrading most of the structural components of the ECM, which is sequentially synthesized, assembled and replaced during corneal development. MMPs may also target other proteinases, latent growth factors, cell surface receptors, and cell adhesion molecules [Sternlicht and Werb, 2001; Egeblad and Werb, 2002]. The MMPs are key players in the diverse tissue remodeling events of embryonic and postnatal development, in adult remodeling processes such as wound repair and angiogenesis, and in a wide variety of pathologies, including arthritis, cancer, and corneal ulceration [Sato et al., 1994; Nagase, 1998; Fini et al., 1998a,b].

While most MMPs are secreted, membrane-type-MMPs (MT-MMPs) are localized on the cell surface, where they are involved in pericellular proteolysis [Sato et al., 1996; Holmbeck et al., 1999]. The presence of MMPs on the cell surface is thought to trigger multiple proteinase cascades associated with cell growth, migration, and morphological changes in pericellular tissues [Nagase and Woessner, 1999; Seiki, 1999; Kajita et al., 2001]. Active MT-MMPs can degrade ECM molecules such as collagen types I, II and III, fibronectin, laminin 1 and 5, vitronectin, fibrinogen, and aggrecan [Ohuchi et al., 1997; d'Ortho et al., 1997; Buttner et al., 1998; Fosang et al., 1998; Koshikawa et al., 2000; Egeblad and Werb, 2002]. The proteolytic removal of the MT-MMP prodomain by furin-like enzymes in the *trans*-Golgi network or by plasmin at the cell surface is required for MT-MMP-induced activation of pro-MMP-2 [Sato et al., 1994; Strongin et al., 1995; Okumura et al., 1997] and pro-MMP-13 [Knauper et al., 1996], indicating that MT-MMP could involve in degradation of type IX collagen. In addition, the activities of MMPs are tightly controlled by the members of the tissue inhibitors of metalloproteinases (TIMP) family [Alexander et al., 1996]. Fitch et al. [2005] demonstrated that treatment of cultured 4.5- to 5-day anterior eye tissues with MT3-MMP and MMP-2 together or TIMP-2 alone resulted in alterations of corneal swelling and cell invasion. In addition, type IX collagen is known to be a specific substrate for MMP-13 [Wu et al., 1991; Knauper et al., 1997], suggesting that MMP-13 could potentially mediate PS swelling. However, no previous work has specifically investigated MMP and TIMP protein levels in the developing cornea in vivo.

Migrating NC cells interact with a variety of ECM molecules via adhesion molecules for proper orientation and development [Perris, 1997; Corbel et al., 2000; Doane et al., 2002]. One such adhesion molecule is the HA receptor, CD44, which primarily binds HA, but can also bind to other ECM components, including collagens, fibronectin, laminin, fibrin, and chondroitin sulfate [Naor et al., 1997]. The gene encoding CD44 includes 20 exons, and numerous different isoforms may be produced by alternative splicing [Thorne et al., 2004]. Different combinations of 10 variant exons (v1–v10) encoding parts of the extracellular domain, as well as variations in glycosylation of the extracellular domain, generate many isoforms of different molecular sizes. Normal cells usually

express standard CD44 (CD44s), which lacks the entire group of variant exons [Naor et al., 1997; Kajita et al., 2001], whereas variants are often seen under disease pathologies. For example, CD44v6 is highly expressed in advanced stage carcinomas [Wielenga et al., 1993; Kaufmann et al., 1995]. Studies have shown that sequential proteolytic cleavages of the ectodomain and the intramembranous domain of CD44 play critical roles in the efficient invasion of cancer cells and metastasis [Okamoto et al., 2002; Nagano et al., 2004], as well as in various disease pathologies [Isacke and Yarwood, 2002]. CD44 ectodomain shedding from the cell surface, with the accompanying promotion of cell migration, has been reported in cells treated with MT3-MMP, as well as a member of the "a disintegrin and metalloproteinases" (ADAM) family, ADAM10 [Kajita et al., 2001; Mori et al., 2002; Nagano et al., 2004]. Members of the ADAM family are membrane-anchored glycoproteins with diverse functions. In particular, ADAM10-deficient mice die in the early embryogenesis due to multiple defect, including a small optic cup [Hartmann et al., 2002].

At present, very little is known about the molecular mechanisms that regulate the migration of NC-derived cells into the HA-enriched primary and secondary stroma, or the pathways underlying matrix remodeling during corneal development. Although CD44-HA interactions have been implicated in cell adhesion and migration, wound healing, tumor cell growth, and metastasis [Thorne et al., 2004], and expression of the CD44v6 variant isoform and its ectodomain cleavage has been implicated in tumor invasion and metastasis, no previous work has examined CD44 expression during corneal development. Here, we examined the temporal and spatial expression of candidate MMPs and TIMPs during corneal development, and further sought to elucidate their functional mechanisms in relation to CD44 variant cleavage. Our findings show for the first time that tightly regulated MMP/TIMP expression is involved in the regulation of matrix swelling, cell migration, and corneal maturation during ocular development in the chick.

MATERIALS AND METHODS

Reagents

The monoclonal anti-CD44v6, anti-MMP-2, and anti-MMP-13 antibodies, along with the polyclonal anti-ADAM10 and anti-TIMP-1 antibodies, were obtained from Chemicon (Temecula, CA). The monoclonal HSP70 and polyclonal MT3-MMP antibodies were purchased from Calbiochem (San Diego, CA). The polyclonal TIMP-2 antibody was purchased from Sigma (St. Louis, MO). The HRP-conjugated secondary antibodies were obtained from Santa Cruz (Santa Cruz, CA). The broad-spectrum synthetic inhibitor of MMP, GM6001, was obtained from Chemicon. Type I collagenase was obtained from Worthington (Freehold, NJ). The tissue culture media (F-12 and DMEM) and fetal calf serum (FCS) were obtained from Gibco (Grand Island, NY). Although all of antibodies used in this study had been generated against mammalian (mostly human) antigens, most of these antigens showed 80% or higher sequence homologies with the relevant chick proteins, and in some cases, the manufacturer's information indicated good cross-reactivity to the chick, suggesting that these antibodies were acceptable choices for the present work. In addition, we included positive control samples with known cross-reactivity in our experiments.

Tissue

Fertilized White Leghorn chicken eggs were incubated at 38°C in a humidified incubator. Corneas were dissected from the embryos at ED 5, ED 7, ED 10, ED14, ED 18, and 2 days post-hatching (P2). All corneas were immediately frozen in liquid nitrogen and maintained as such until protein extraction. For immunohistochemistry, eyes were mounted in embedding medium (Sakura Finetek, Torrance, CA), rapidly frozen in propane chilled with liquid nitrogen (−160°C) and stored at −80°C until use.

Tissue Extraction

Aliquots of frozen corneas (approximately 500 corneas at ED 5 and 50 corneas at ED 18) were placed in Eppendorf tubes containing RIPA buffer (1% Nonidet-P 40, 0.1% SDS, 0.1% deoxycholic acid, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM EDTA, 150 mM NaCl, and protease inhibitors in 50 mM Tris-HCl, pH 7.4). The solution was homogenized with a pellet pestle motor (Sigma Diagnostics, St. Louis, MO), the homogenates were centrifuged at 13,000 rpm for 30 min at 4°C, and the supernatants were collected. The total protein

concentration in each supernatant was determined using the BCA protein assay (Pierce, Rockford, IL).

Corneal Cell Culture

Corneal cell culture was carried out according to the previously described protocol [Cai and Linsenmayer, 2001]. In brief, 300 corneas dissected from developing embryos at ED 12 or ED 14 were treated with 0.5% dispase in PBS for 1 h at 4°C, and the epithelial layers were gently scraped away with a scalpel. The epithelia were rinsed with PBS and further digested in 0.25% trypsin at 37°C for 5 min, while the stromas were incubated with type I collagenase (5 mg/ml) at 37°C for 3 h to induce the release stromal fibroblasts. Both cell types were grown in a 1:1 mixture of DMEM and F-12 medium supplemented with heat-inactivated 10% FCS, 1% chicken serum, 5 μg/ml insulin, 10 ng/ml human recombinant EGF, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 200 μg/ml gentamicin (all purchased from Gibco). For experiments investigating whether CD44v6 shedding was regulated by the observed MMP activities, the cells were incubated with 20 μM of GM6001 for 24 h. The positive control HT29 human colon carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA), and grown to monolayers and maintained as previously described [Singh et al., 2001].

Preparation of Conditioned Medium

In order to analyze secreted protein levels, equal densities of freshly isolated cells were plated in culture dishes with medium containing 10% FCS. This medium was replaced with serum-free medium the following day, and the cells were allowed to condition the serum-free medium for 24 h. The conditioned media were collected and centrifuged briefly for removal of cell debris, and equal volumes of the supernatant were divided into aliquots (usually 500 μl). Secreted proteins were precipitated from each aliquot using 10% ice-cold trichloroacetic acid (TCA), and the precipitates were washed twice with 100% acetone for removal of residual TCA. The precipitates were air-dried, dissolved in RIPA buffer and stored at −20°C until use.

Western Blot Analysis

Western blot analysis was performed using standard techniques. Equal amounts of protein lysates (30 μg) were separated by SDS–PAGE (7.5 or 10%) under nonreducing conditions, and the proteins were electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h to minimize nonspecific binding. The primary antibodies against MMP-2, MMP-13, MT3-MMP, ADAM10, TIMP-1/TIMP-2, and CD44v6 were diluted in TBS-T containing 5% dried milk and then incubated with the membrane for 1 h. The primary antibodies were detected with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG or goat anti-mouse IgG, and the results were visualized with an enhanced chemiluminescence (ECL kit, Amersham Biosciences) followed by exposure to X-ray film. Each experiment was repeated three times.

RT-PCR Analysis of MMP-13 mRNA Expression

RT-PCR was performed and analyzed as previously described [Fitch et al., 1998, 2005]. In brief, RNAzol B was used to isolate total RNA from 100 developing corneas at ED 5 and ED 7, as well as from 30 developing costal (hyaline) rib cartilage samples (ED 14), and 1 μg of total RNA was used to generate oligo(dT)18-primed cDNA with an RT-PCR kit (Clontech, Palo Alto, CA), according to the manufacturer's instructions. Amplification of MMP-13 was conducted using the previously described primers (#782–801, TTTGGGCTATGAATGGCTAT and #1098–1117, TAGTATGCAGGATGCGGACA) [Fitch et al., 1998, 2005] and the following reaction conditions: one cycle of 94°C for 2 min, 58°C for 30 s and 72°C for 1 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s and a final elongation at 72°C for 10 min. This method resulted in amplification of a single major cDNA fragment (336 bp), as visualized by agarose gel electrophoresis.

Immunoprecipitation

Lysates (700 μg) from 500 developing corneas at ED 7 were incubated with a monoclonal CD44v6 antibody for 12 h at 4°C. The mixture was then incubated with Protein A-coated agarose beads (Santa Cruz Biotechnology) for 3 h with constant rocking at 4°C. The beads were washed three times with RIPA buffer to remove nonspecific binding, followed by centrifugation.

The bound proteins were eluted by boiling for 5 min in SDS–PAGE sample buffer containing 1% 2-mercaptoethanol, and then separated by 7.5% SDS–PAGE. The resolved proteins were transferred to a nitrocellulose membrane, which was then probed with an antibody against ADAM10. The signal was detected with a peroxidase-conjugated secondary antibody and visualized with an ECL kit.

Immunohistochemistry

Cryosections (7 μm) were cut, placed onto slides previously pretreated with a 0.01% poly-L-lysine solution in deionized water (Sigma Diagnostics), and kept at −20°C until use. For staining, air-dried sections were fixed in 4% paraformaldehyde for 10 min at room temperature. Heat-induced antigen retrieval, especially for post-fixation CD44v6 staining, was performed as follows. For recovery of the antigenic determinant of CD44v6, sections were submerged into 0.01 M citrate buffer, pH 6.0, and incubated at 95°C for 25 min. Endogenous peroxidase activity was inactivated by incubation in 0.3% H_2O_2 in methanol for 10 min, and the sections were processed for immunoperoxidase localization using antibodies against CD44v6, ADAM10, and MT3-MMP. Specific binding of the primary antibodies was visualized with biotin-labeled secondary antibodies and the ABC reagent kit (Vector Labs, Burlingame, CA) according to the manufacturer's instructions. Staining was also visualized using a Vector NovaRED substrate kit (Vector Labs). Sections were observed with a Nikon bright-field microscope, and digitized images were obtained using a SPOT digital camera.

RESULTS

CD44v6 Expression and Cleavage is Regulated by MMPs During Corneal Development

Immunohistochemistry (Fig. 1A) revealed that, at ED 5, CD44v6 was highly localized in the periocular tissues surrounding the cornea, and also in head mesenchymal-derived NC cells from the periocular tissues invading PS located between the corneal and lens epithelium. The corneal PS was previously shown to be a product of the corneal epithelium [Hay and Revel, 1969; Hendrix et al., 1982; Svoboda et al., 1988; Doane et al., 1996, 2002; Fitch et al., 1998; Akimoto et al., 2002; Marchant et al., 2002]. At ED 7, CD44v6 immunoreactivity was also detected strongly in the epithelium, especially in the NC-derived stromal cells invading into the posterior region of the swollen secondary stroma, but only weakly in the endothelium. NC-derived stromal cell migration continued through ED 10, and formation of the secondary stroma was completed at ED 14, when cell migration ceased and the hydrated stroma began to condense [Siegler and Quantock, 2002]. Thereafter, the secondary cornea underwent significant structural, compositional, and transparency changes [Trelstad and Coulombre, 1971; Siegler and Quantock, 2002]. At ED 10, CD44v6 immunoreactivity was ubiquitous in the epithelium, stroma, and endothelium, with higher levels seen in the anterior stroma just underneath the epithelium, compared to the posterior stroma. However, between at ED 14 and ED 18, CD44v6 was strongly detected in the maturing corneal epithelium, but gradually decreased throughout the stromal layer. At P2, the signal was strongly detected in the basal epithelium of the cornea, but not in the stromal layer or endothelium. Taken together, these results indicated that CD44v6 was expressed in spatially distinct patterns in developing chick corneas, suggesting that the migration of NC cells into the corneal stroma might be associated with CD44v6 activity.

Next, we used Western blotting to examine the presence of CD44v6 cleavage in lysates of developing corneas. The standard form of CD44 (CD44s), which did not contain any variant exon products, was ≈85-kDa [Okamoto et al., 2002; Nagano et al., 2004]. Therefore, the bands above ≈85-kDa were identified as CD44v6, and those below ≈85-kDa were identified as CD44v6 cleavage products. Interestingly, five major bands corresponding to CD44v6 were detected (Fig. 1B). Of them, the ≈110-, ≈97-, and ≈90-kDa bands were likely to represent un-cleaved forms of CD44v6, while the ≈74- and ≈62-kDa bands may represent the cleaved forms of CD44v6. The presence of different molecular sizes of CD44v6 may be due to variations in glycosylation of the extracellular domain. These five bands were most notably detected in the early developing corneas between ED 5 and ED 14, whereas the ≈74- and ≈62-kDa bands were not detectable at ED 18 and P2, but the un-cleaved forms of CD44v6 were barely detectable at ED 18 and P2. This data suggesting that

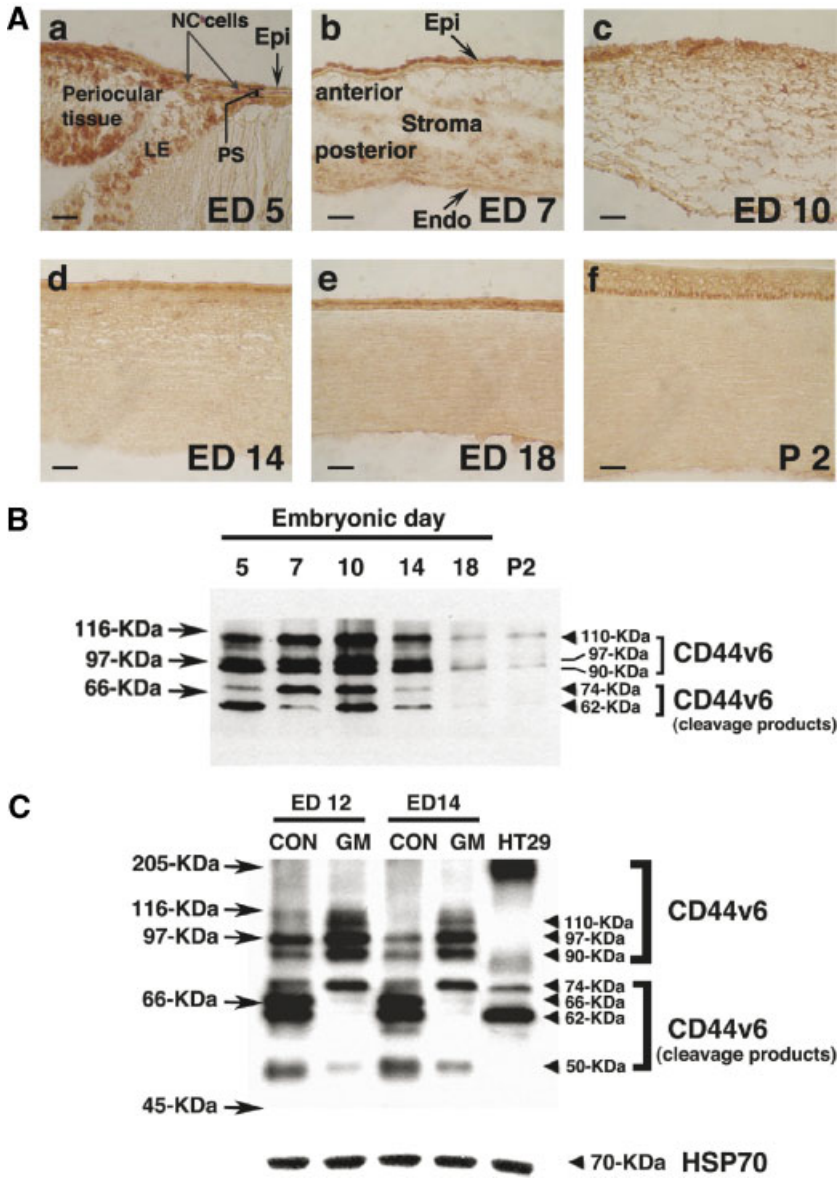

Fig. 1. Temporal and spatial expression patterns of CD44v6 and its shedding forms in developing corneas, and the involvement of MMP activity. **A**: Immunolocalization of CD44v6 in developing corneas at ED 5 (a), ED 7 (b), ED 10 (c), ED 14 (d), ED 18 (e), and P2 (f). CD44v6 was highly expressed in periocular tissues surrounding the cornea, in the corneal and lens epithelium (LE), and in the NC-derived stromal cells that had penetrated into the PS by ED 5. Staining was also highly detected in the corneal epithelium (Epi) at ED 7, but only weak signals were detected in the endothelium (Endo). Interestingly, CD44v6 expression was prominent in the invading NC-derived stromal cells, especially those in the posterior region of the swollen stroma. At ED 10, CD44v6 expression was detected ubiquitously in the cornea. At ED 14 and ED 18, CD44v6 expression was strongly detected in the maturing corneal epithelium, but its expression gradually decreased in the stromal layer. Note that the signal was strongly detected only in the basal epithelium by P2, but not in the stromal layer or endothelium. Scale bar, 20 μm. **B**: Equal amounts of corneal lysates (30 μg) were resolved under nonreducing conditions and analyzed for CD44v6 expression by Western blot analysis. Note that the ≈110-, ≈97-, and ≈90-kDa bands presumably due to different glycosylation rate were detected as major forms and were expressed in the developing corneas for up to 14 days. Thereafter, all bands dramatically decreased. The standard form of CD44 (CD44s), which does not contain any variant exon products, is ≈85-kDa. Therefore, the bands above ≈85-kDa were identified as CD44v6, and those below ≈85-kDa were identified as CD44v6 cleavage products. **C**: Primary cultured stromal cells were treated with GM6001 as described in ''Materials and Methods.'' Equal amounts of cultured stromal cell lysates (30 μg) were analyzed for CD44v6 shedding (ectodomain cleavage), which was markedly inhibited in GM6001 cultures versus controls. In contrast, the un-cleaved CD44v6 bands (≈110-, ≈97-, and ≈90-kDa) were stronger in the GM6001-treated cultures than in control cultures. Lysates of HT29 human colon carcinoma cells were used as positive controls for CD44v6, and HSP70 was used as a loading control. The molecular weight markers are shown on the left (in kDa), and the presented data are representative of at least three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

CD44v6 cleavage was developmentally regulated in the chick cornea.

To examine whether CD44v6 cleavage was mediated by MMPs in the developing chick cornea, we examined CD44v6 cleavage products in primary cultured stromal cells isolated from ED 12 and ED 14 and treated with 20 μM of GM6001 for 24 h. Western blot analysis revealed that the pattern of the CD44v6 cleavage products in control cultures resembled that from developing corneas (Fig. 1C), with the addition of two additional cleaved bands at ≈66- and ≈50-kDa. Cells treated with GM6001 showed high levels of CD44v6 immunoreactivity, but had significantly deceased levels of the ectodomain cleavage products, including the ≈66- and ≈50-kDa bands, as compared to the control cultures (Fig. 1C). HT29 human colon carcinoma cells expressed high levels of CD44v6 [Mitchell et al., 1996; Singh et al., 2001]. As expected, ≈74- and ≈62-kDa bands were detected in both chick and human samples, suggesting that CD44v6 antibody generated against human CD44v6 has a cross reactivity to the chick. Taken together, these data indicated that CD44v6 ectodomain cleavage in the developing chick cornea was mediated by MMP activity.

Expression Patterns of MMP-2, MMP-13, TIMP-1 and TIMP-2 Proteins During Corneal Development

To examine the expression patterns of MMPs that might contribute to cell migration or matrix remodeling during corneal development, we performed Western blot analysis. We tested for protein expression of MMP-2, which was previously identified in early developing chick corneas at ED 7 [Fitch et al., 1998, 2005], and MMP-13, since this MMP was known to cleave type IX collagen, which disappears rapidly at ED 7 during chick corneal development [Fitch et al., 1998]. Our results revealed high levels of MMP-2 at a 62-kDa, a size consistent with that reported for its proteolytically activated form [Fitch et al., 1998]. Consistent with the previous report, this active protein was easily detectable at ED 7. In addition, our results revealed for the first time that active MMP-2 was expressed throughout corneal development (Fig. 2A), suggesting a possible role for MMP-2 in the complete removal of partially cleaved type IX collagen and ECM remodeling.

Interestingly, probing of the same lysates for MMP-13 revealed a very different temporal pattern. High levels of proteolytically active (≈50-kDa) MMP-13 protein were detected at ED 7, but these levels gradually decreased thereafter. Some MMP-13 could be detected at ED 10, but not at later time points (Fig. 2A). Previous studies have identified high-level MMP-13 expression in articular cartilage, where it contributes to collagen catabolism, and is highly induced during chondrocyte hypertrophy in chick [D'Angelo et al., 2000; Fitch et al., 2005]. In this study, we compared MMP-13 levels in lysates from developing chick cornea versus those from developing chick costal (hyaline) rib cartilage. The truncated (active) form of the MMP-13 protein (≈50-kDa) was strongly detected in developing corneas at ED 7 and cartilage samples at ED 14 (Fig. 2B). In contrast, the full-length pro-form of MMP-13 (≈59-kDa) was detected in the developing cartilage at ED 14, but not in developing corneas at ED 7 (Fig. 2B), indicating that most secreted pro-form of MMP-13 especially in developing corneas was converted to active form. To examine whether the presence of MMP-13 protein in the developing tissues was the result of MMP-13 gene expression by resident cells, MMP-13 mRNA expression was assayed by RT-PCR. As expected, MMP-13 mRNA was expressed in both developing corneas at ED 5 and ED 7, as well as in developing cartilage at ED 14 (Fig. 2C). Taken together, our data revealed for the first time that active MMP-13 was expressed early in corneal development, suggesting a possible role for MMP-13 in degradation of type IX collagen.

We then used Western blotting to assess the presence of TIMP-1 and TIMP-2 in developing corneas (Fig. 2A). TIMP-1 protein expression was not detectable in ED 5 or ED 10 corneas (Fig. 2A). In contrast, TIMP-1 could be detected in late developing corneas after ED 18, and its corneal expression was maximal at P2. Interestingly, two bands corresponding to TIMP-2 protein were constitutively present throughout corneal development (Fig. 2A). These findings collectively suggesting that MMPs were expressed and active in the early developing cornea, where they may cleave type IX collagen and other ECM molecules, but that their expression and/or activity is more restricted at later time points during corneal development.

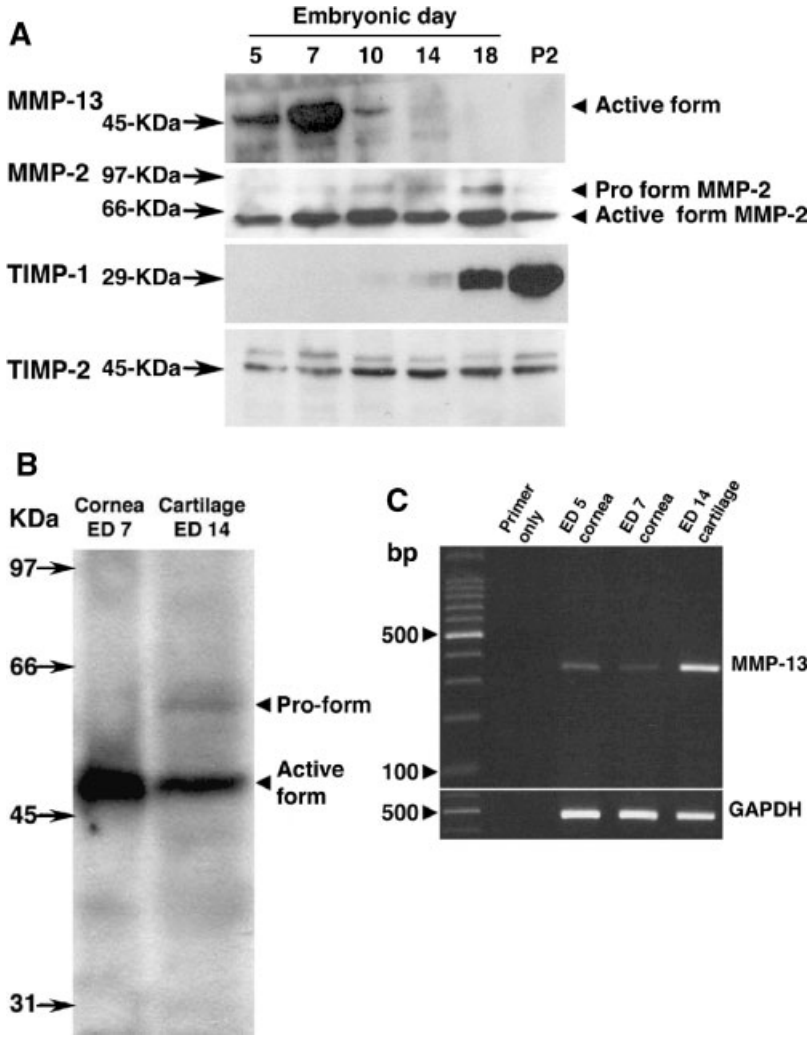

Fig. 2. Differential expression patterns of MMP-2, MMP-13, TIMP-1 and TIMP-2 in developing corneas. **A**: Samples were extracted from whole developing corneas at ED 5, ED 7, ED 10, ED 14, and ED 18, and at P2. Equal amounts of protein (30 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) under nonreducing conditions and analyzed for MMP-2 and MMP-13 protein expression by Western blot. It is notable that the 50-kDa form of MMP-13 was strongly expressed in the ED 7 developing corneas, whereas the active form of MMP-2 (≈62-kDa) was constitutively expressed throughout corneal development. Note that TIMP-1 (≈29-kDa) was not detected in the developing corneas between at ED 5 and ED 10, but could be seen in late developing corneas between ED 14 and P2. In contrast, TIMP-2 (≈45-kDa doublet) was constitutively expressed throughout corneal development. The data shown are representative of at least three independent experiments. **B**: Western blot analysis of developing corneas at ED 7 and developing costal (hyaline) rib cartilage at ED 14. Note that the ≈50-kDa MMP-13 (active form) was expressed in both developing corneas and cartilage, whereas the 59-kDa pro-form of MMP-13 was weakly detected only in the cartilage. **C**: RT-PCR analysis of MMP-13 mRNA expression in developing corneas. MMP-13 mRNA was detected in developing corneas at ED 5 and ED 7, and in developing cartilage at ED 14. GAPDH was used as the loading control, and the left-hand lane shows DNA size markers.

Temporal and Spatial Regulation of MT3-MMP Expression During Corneal Development

Previous studies have shown that MT-MMPs form a complex with CD44 and cleave the ectodomain at the leading edge of migrating cells, facilitating cell migration and reorganization of the ECM [Kajita et al., 2001; Mori et al., 2002; Cichy and Pure, 2003; Thorne et al., 2004]. Furthermore, the cleaved extracellular region was shown to be secreted and sequestered into the ECM [Thorne et al., 2004], and inhibition of MT-MMP was found to suppress CD44-dependent cell migration [Okamoto et al., 1999]. Based on these previous reports, we examined whether MT3-MMP was expressed in the developing corneas by immunohistochemistry (Fig. 3A). In the developing cornea at ED 5,

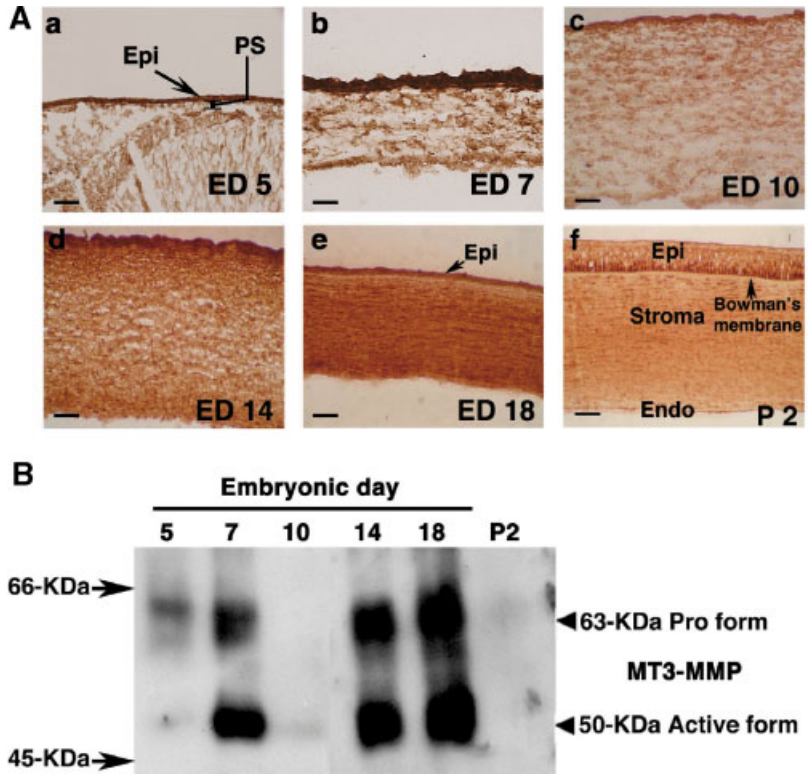

Fig. 3. Temporal and spatial expression of MT3-MMP in developing corneas. **A**: Immunolocalization of MT3-MMP in developing corneas at ED 5 (a), ED 7 (b), ED 10 (c), ED 14 (d), ED 18 (e), and P2 (f). In the developing cornea at ED 5, MT3-MMP was strongly detected in the epithelium and the PS. At ED 7, MT3-MMP was highly detected in the epithelium and in all invading NC-derived stromal cells. At ED 10, the staining in the epithelium was relatively weak compared to that ED 7, and the anterior stroma showed more staining than the posterior stroma. At ED 14, a strong signal was detected in the epithelium, the anterior region of the stroma, the posterior stroma and the endothelium. By ED 18, the MT3-MMP signal was present in all corneal layers except for Bowman's membrane. At P2, the signal was strongly detected in the basal epithelium, but not in the superior epithelium. Moreover, MT3-MMP was strongly detected in the anterior and central regions of the stroma, but only a weak signal was detected in the posterior stroma. Scale bar, 20 μm. **B**: Detection of MT3-MMP by Western blot analysis. High levels of both the pro- (≈63-kDa) and active (≈50-kDa) forms of MT3-MMP were observed in the developing corneas at ED 7, ED 14, and ED 18, but not in the corneas at ED 10 or P2. Our immunohistochemistry experiments indicated low-level expression of MT3-MMP at ED 10 and P2; presumably, this expression was below the level of detection by Western blotting under our conditions. The data shown are representative of at least three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

MT3-MMP was strongly detected in the epithelium and the PS. At ED 7, the highest levels of MT3-MMP were detected in the epithelium. At ED 10, strong MT3-MMP expression was sustained in the epithelium and a weak signal was detected in the stroma. At ED 14, high levels of MT3-MMP were detected throughout the developing cornea, and by ED 18, strong MT3-MMP immunoreactivity was observed in all aspects of the cornea, except for Bowman's membrane. At P2, MT3-MMP expression was weakly detected throughout the cornea, with the exception of the basal layer of the epithelium.

Based on the biphasic expression patterns observed for MT3-MMP by immunohistochemistry, we hypothesized that the active form of MT3-MMP might be necessary during the periods of extensive cell migration and corneal maturation. As previous studies had shown that MT3-MMP was produced as an inactive zymogen that could be activated by furin-like convertases [Shofuda et al., 1997; Jung et al., 2002; Zhao et al., 2004], we examined the presence of active forms of MT3-MMP in the developing corneas by Western blotting. Consistent with our hypothesis, high levels of the active (≈50-kDa) and pro (≈63-kDa) forms of MT3-MMP were observed in the developing corneas, especially at ED 7, ED 14, and ED 18 (Fig. 3B). In addition, our immunohistochemistry experiments revealed weak MT3-MMP immunoreactivity at ED 10 and P2. Western blotting did not

detect MT3-MMP protein expression at these stages. However, relatively short X-ray exposure times were used to analyze the very strong Western blot signals at ED 7, ED 14, and ED 18, meaning that weak signals at ED 10 and P2 might have been missed. Thus, our data collectively suggest that active MT3-MMP may participate in ectodomain cleavage of CD44v6 as well as ECM remodeling in the pericellular environment of the developing chick cornea.

Temporal and Spatial Regulation of ADAM10 Expression During Corneal Development

Since studies have shown that the extracellular domain of CD44 could be cleaved by ADAM10 [Kajita et al., 2001; Mori et al., 2002; Nagano et al., 2004], and depletion of ADAM10 markedly suppressed the migration of CD44-dependent cancer cells on HA [Nagano et al., 2004], we examined the possible involvement of ADAM10 in corneal development. Immunohistochemical analysis of ADAM10 expression was performed in the developing cornea (Fig. 4A) revealed that at ED 5, ADAM10 was strongly detected in the epithelium and PS. At ED 7, ADAM10 was highly detected in the epithelium, but was weakly detected in the anterior stroma. At ED 10, ADAM10 expression was highly detected in all NC-derived stromal cells and epithelium, as well as in the endothelium. However, by ED 14, ADAM10 expression was dramatically reduced in the epithelium, but a very strong signal was retained in all regions of the stroma. By ED 18, ADAM10 expression was also weakly detected in the epithelium, but a strong signal persisted in the stroma. Notably, at P2, ADAM10 expression was no longer detected in the epithelium and Bowman's membrane, but a very weak signal detected in the stroma.

We then examined whether active and secreted forms of ADAM10 were present in the developing corneas. ADAM10 is expressed as an inactive pro-form (97-kDa) that is processed to a shorter, active form (68-kDa) [Alexander et al., 1996; Nagano et al., 2004; Sahin et al., 2004]. Although two alternative spliced forms of mRNA encoding ADAM10 (4.5 and 2.8 kb) have been identified in several developing chick tissues [Hall and Erickson, 2003], only a single 82-kDa protein was detected by Western blot analysis of these tissues [Hall and Erickson, 2003]. In contrast, our Western blot analysis identified three major forms of ADAM10 protein in developing chick corneas ($\approx$82-, $\approx$57-, and $\approx$55-kDa), and showed time-dependent differences in their expression levels (Fig. 4B). All three forms of ADAM10 were expressed in ED 5, ED 7, and ED 10 corneas, whereas the $\approx$82- and $\approx$57-kDa proteins were not detected in ED 14, ED 18, and P2 corneas, which showed only faint bands corresponding to the $\approx$55-kDa of ADAM10 protein. Although ADAM10 was membrane-anchored glycoprotein, ADAM10 was present in the pericellular ECM of the developing stroma between ED 10 and ED 18 by immunohistochemistry. Therefore, we hypothesized that the $\approx$57- and $\approx$55-kDa proteins might be secreted and sequestered into the ECM of the stroma. To examine this, we assayed secretion of ADAM10 to conditioned media collected from primary cultured stromal and epithelial cells isolated at ED 12. Consistent with our hypothesis, the $\approx$57- and $\approx$55-kDa proteins were present in the collected conditioned media (Fig. 4C). We then sought to determine whether ADAM10 was able to complex with CD44v6, using monoclonal anti-CD44v6 immunoprecipitation of lysates prepared from ED 7 corneas. As expected, the active mature form of CD44v6 ($\approx$68-kDa) could be detected with an ADAM10 antibody upon Western blotting of the immunoprecipitates (Fig. 4D), suggesting that in addition to its cleavage by MT3-MMP, the CD44v6 ectodomain could also be cleaved via ADAM10 in developing chick corneas.

DISCUSSION

Although researchers have long speculated that MMPs may play an important role in corneal development, previous studies have provided only correlative connections between MMP/TIMP function and corneal development in vivo. Here, we demonstrated for the first time that the expression levels of MMP-2 and MMP-13, MT3-MMP, ADAM10 and TIMP-1 and TIMP-2 were temporally and spatially regulated in the developing cornea, directly suggesting that corneal development in vivo is mediated via MMP-dependent mechanisms.

MMPs and TIMPs May Interact to Mediate Cleavage of Type IX Collagen, Which is Associated With Corneal Swelling, Cell Migration, and ECM Remodeling

MMPs are typically synthesized as inactive proenzymes, and their protease abilities

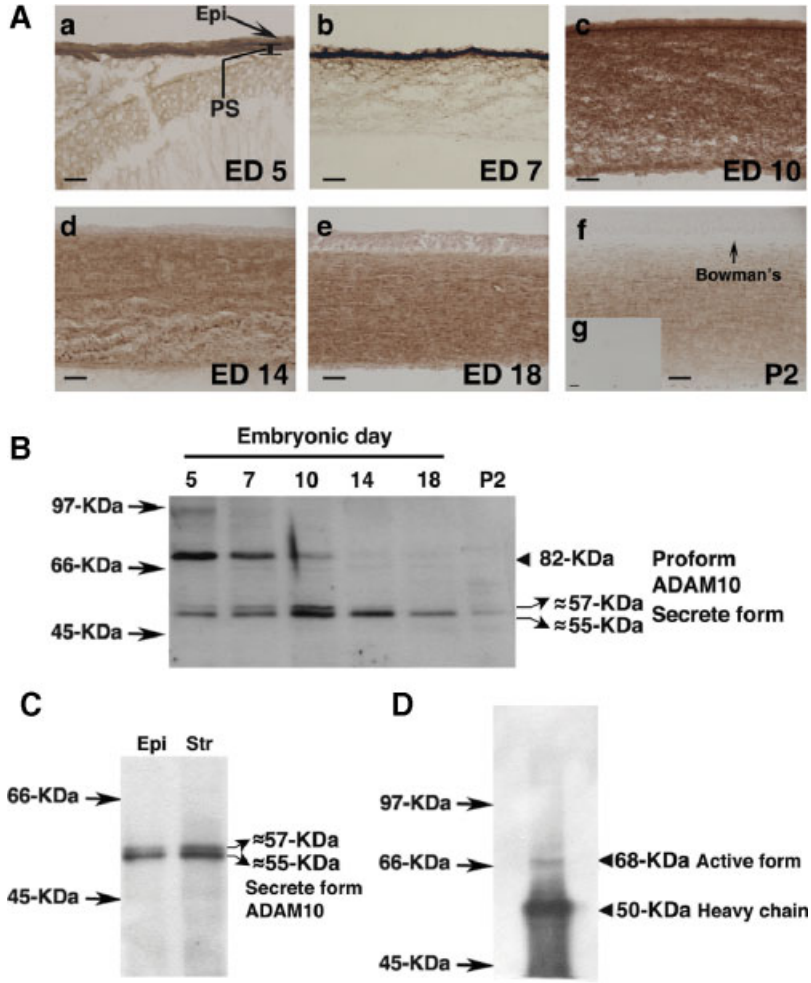

Fig. 4. Temporal and spatial expression of ADAM10 in developing corneas, and its complex formation with CD44v6. **A**: Immunolocalization of ADAM10 in developing corneas at ED 5 (a), ED 7 (b), ED 10 (c), ED 14 (d), ED 18 (e), and P2 (f). At ED 5, ADAM10 was strongly detected in the single-layered epithelium as well as PS. At ED 7, strong signals were detected in the epithelium, but relatively weak signal was detected in the anterior stroma. At ED 10, abundant ADAM10 expression was seen throughout the cornea. At ED 14, a strong signal was detected in the stroma, and weak signals were detected in the epithelium and endothelium. At ED 18, a strong signal was detected in the stroma, but the signal was weakly detected in the epithelium and Bowman's membrane. At P2, ADAM10 signal was no longer detected in both epithelium and Bowman's membrane, but a weak signal was detected throughout the entire stroma. In all cases, negative controls (g) incubated without the primary antibody failed to show staining. Scale bar, 20 μm. **B**: Detection of ADAM10 by Western blot, which showed differential expression of the dominant ≈82-kDa, and ≈57- and ≈55-kDa bands during corneal development. All three bands were detected in ED 5, ED 7, and ED 10 corneas, but only the ≈55-kDa band was detectable in ED 14, ED 18, and P2 corneas. **C**: Detection of ADAM-10 secretion from primary cultured epithelial and stromal cells isolated from ED 12. Both the ≈57- and ≈55-kDa proteins were present in the collected media. **D**: Lysates (700 μg) from the developing cornea at ED 7 were immunoprecipitated with the CD44v6 antibody. Note that active ADAM10 (≈68-kDa) was detected by Western blot analysis against ADAM10, suggesting that CD44v6 shedding might be mediated by active ADAM10. The molecular weight markers are shown on the left (in kDa), and the presented data are representative of at least three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

are dependent upon the presence of appropriate activating mechanisms [Sato et al., 1994; Kashiwagi et al., 2001]. We herein showed for the first time that high levels of MMP-13 mRNA and the active form of MMP-13 were present in the early developing corneas (Fig. 2A–C). Notably, the temporal expression pattern of MMP-13 correlated with the disappearance of type IX collagen, which is known to be a specific substrate of MMP-13 [Wu et al., 1991; Knauper et al., 1997]. Both the membrane-bound and secreted forms of ADAM10 were detected in the cornea throughout its development (Fig. 4). As the catalytic capability of active MMPs are

tightly controlled by the TIMPs [Alexander et al., 1996], we examined TIMP function in the developing chick cornea. We found that TIMP-1, which can inhibit the action of MMP-13 and ADAM10 [Amour et al., 2000; Kashiwagi et al., 2001; Egeblad and Werb, 2002], was not expressed during the early stages of corneal development (Fig. 2A), suggesting that MMP-13 and ADAM10 are fully active at this point. In contrast, TIMP-1 expression increased later in corneal development (Fig. 2A), suggesting that the accumulation and remodeling of ECM molecules in the maturing cornea may be triggered by inhibition of MMP activity. In addition, the TIMPs are multifunctional proteins having diverse capabilities for modulating growth factor activity, steroidogenesis and cell morphology [Gomez et al., 1997; Bond et al., 2000; Hoegy et al., 2001]. Therefore, it is likely that TIMPs may promote epithelial growth through other pathways in addition to MMP inhibition.

Our results revealed both active MMP-2 and TIMP-2 proteins were constitutively present throughout corneal development (Fig. 2A). On the other hand, high levels of the active form of MT3-MMP were detected in developing corneas at ED 7, ED 14, and ED 18 (Fig. 3B). It is well known that higher levels of TIMP-2 can inhibit the activities of MT3-MMP and MMP-2 [Strongin et al., 1995; Knauper et al., 1996; Toth et al., 2003], and high doses of TIMP-2 were shown to block type IX collagen degradation and stromal swelling in organ-cultured early developing corneas [Fitch et al., 2005]. In the latter case, it seems likely that the high doses of TIMP-2 inhibited MMP-2 activation by saturating the MT3-MMP activity required for prodomain cleavage of MMP-2 and MMP-13 [Strongin et al., 1995; Okumura et al., 1997; Buttner et al., 1998]. Thus, TIMP-2 has a bi-functional role in the regulation of MMP-2 activity [Butler et al., 1998]. Although future studies will be required to clarify the precise molecular mechanism(s) involved, our present findings seem to suggest that the levels of TIMP-2 detected in normal developing corneas in vivo (Fig. 2A) are low enough that MMP-2 is activated, not repressed, throughout corneal development. Furthermore, [Fitch et al., 2005] demonstrated that type IX collagen completely disappeared after co-treatment with active recombinant MMP-2 and MT3-MMP, but not following treatment with either MMP alone. Thus, the high level expression of active MMP-13 detected in early developing corneas in vivo (Fig. 2A–C), which may be mediated by MT3-MMP-mediated conversion of pro-MMP-13 to active MMP-13 [Cowell et al., 1998; Toth et al., 2003], likely co-operates in the digestion of type IX collagen. In addition, active MMP-2 may assist in the complete removal of partially cleaved type IX collagen. Thus, MMP-13, MMP-2, and MT3-MMP co-operate to cleave the type IX collagen that holds HA in the compact PS, freeing the HA to undergo the hydrostatic swelling required for NC-derived stromal cell invasion during early corneal development.

MT3-MMP and ADAM10 May be Involved in Ectodomain Cleavage of CD44v6, Which Modulates Cell Migration, Proliferation, and ECM Remodeling in the Developing Cornea

While normal cells, including pre-migratory cranial NC cells, usually express the standard form of CD44 (CD44s) [Corbel et al., 2000], variant isoforms, especially CD44v6, are highly expressed by many malignant tumor cells [Wielenga et al., 1993; Kaufmann et al., 1995; Naor et al., 1997; Kajita et al., 2001]. CD44v6 is also expressed in activated normal cells and contributes to disease pathologies, where it modulates cell migration, proliferation, differentiation, and ECM remodeling [Isacke and Yarwood, 2002]. In the present study, we found that migrating NC cells infiltrating into the primary and secondary stroma expressed high levels of CD44v6 (Fig. 1A). During the most active period of cell migration, which occurred up to ED 14, the CD44v6-expressing cells were located in the developing stroma, where they were positioned to mediate cell-ECM interactions (Fig. 1A). CD44v6 expression and cleavage dramatically decreased after ED 14 (Fig. 1B), correlating with the end of stromal cell migration and division, and the disappearance of HA [Trelstad and Coulombre, 1971; Bard and Hay, 1975].

Both ADAM10 and MT3-MMP can cleave the ectodomain of CD44v6, which binds to HA, type I collagen, fibrin and chondroitin sulfate proteoglycans [Kajita et al., 2001; Isacke and Yarwood, 2002]. A synthetic MMP inhibitor was shown to decrease early cardiac NC cell migration [Cai and Brauer, 2002], suggesting that MMP activities may also require for NC cell migration during corneal development. The

present study revealed that both MT3-MMP and ADAM10 were temporally and spatially expressed during corneal development. Furthermore, both the cleaved forms of CD44v6 and the membrane-bound form of ADAM10 (≈82-kDa) were highly detectable in early developing corneas (Figs. 1B and 4B) but not later in development, suggesting that CD44v6 ectodomain cleavage may be mediated at least in part by ADAM10. In support of this notion, we found that the mature form of ADAM10 (≈68-kDa) can form a complex with CD44v6 (Fig. 4D), and that CD44v6 ectodomain cleavage in primary cultured cells was strongly inhibited by treatment with GM6001 (Fig. 1C). Taken together, these findings suggest that direct interaction between CD44v6 and ADAM10 may be involved in cleavage of CD44v6. We postulate that the processing of CD44v6 at the adherent edges of cells is crucial for stimulation of pericellular migration, as it would allow the head mesenchyme-derived-NC cells to detach from periocular tissues for movement into the HA-enriched PS and the secondary stroma. Due to the high levels of CD44v6 expression in the proliferating epithelium during late corneal development (Fig. 1A), it also seems likely that the proliferation in the epithelium is at least partially mediated by up-regulation of CD44v6 expression [Soukka et al., 1997].

Although we observed varied patterns of MT3-MMP expression during the development of various corneal tissues, the active form of MMP-2 appeared to be consistently expressed in all corneal tissues during development. MT3-MMP has been implicated as a possible activator of pro-MMP-2, -9, and -13 [Cowell et al., 1998; Toth et al., 2003], but we found that MT3-MMP was not always co-expressed with the active forms of MMP-2 or MMP-13 in developing corneas. Notably, a previous study showed that MT-MMP generated an MMP-2 activation intermediate that required the involvement of a previously activated MMP-2 for full activation [Deryugina et al., 2001]. Therefore, it seems likely that the activated MMP-2 observed in the early developing corneas was capable of continuing to activate MMP-2 intermediates throughout corneal development. As MT-MMP has been shown to degrade numerous ECM molecules [d'Ortho et al., 1997; Ohuchi et al., 1997; Fosang et al., 1998; Egeblad and Werb, 2002], it seems likely that the observed MT3-MMP may also participate in ECM remodeling during corneal development, in addition to its functions in MMP-2 activation and CD44v6 cleavage. ADAM10 also has a wide variety of substrates and can remodel ECM components besides membrane proteins [Smith et al., 2002]. It is interesting to note that only the secreted forms of ADAM10 (≈55- and ≈57-kDa) were detected during late corneal development, especially in the stroma (Fig. 4A–C). This may suggest that the secreted form of ADAM10 may play an important role in the remodeling of the surrounding pericellular ECM during corneal maturation.

In the developing corneal epithelium, MT3-MMP and ADAM10 expression were differentially regulated. Immunohistochemistry revealed strong expression of MT3-MMP and ADAM10 in the corneal epithelium of early developing corneas (Figs. 3A and 4A). In late developing corneas, however, MT3-MMP was constantly expressed in the proliferating multilayered epithelium, but a weak ADAM10 expression was detected in the maturing epithelium. MMPs can control cellular behavior in remodeling tissue by activating latent forms of cytokines and growth factors, thus increasing their bioavailability [Yu and Stamenkovic, 2000; Karsdal et al., 2002]. The activation of latent TGF-β can be mediated by MT-MMP [Mu et al., 2002]. Therefore, it seems likely that MT3-MMP activity is associated with the activation of cytokines and growth factors that will affect proliferating and migrating epithelial cells.

In summary, we herein showed that MMP-13 mRNA and protein expression could be detected in early developing corneas, but TIMP-1 was not, suggesting that MMP-13 could mediate cleavage of type IX collagen, which is required for matrix swelling during corneal development. We further showed that CD44v6 ectodomain cleavage occurred in an MMP-dependent manner in the developing chick cornea, and found that the expression patterns of MT3-MMP and ADAM10 suggest that these MMPs may be involved in CD44v6 ectodomain cleavage, cell migration, cell proliferation, and ECM remodeling throughout corneal development. These findings collectively support our hypothesis that corneal development in vivo is mediated by the temporal and spatial regulation of multiple MMPs (e.g., MMP-13, MMP-2, MT3-MMP, and ADAM10) and TIMPs (e.g., TIMP-1 and TIMP-2).

ACKNOWLEDGMENTS

This work was supported by Korea Research Foundation (KRF-2003-070-C00033), the Kyungpook National University Research Fund (2002), NIH grant R01 EY12651 and a Research to Prevent Blindness unrestricted grant (to the University of Miami). MEF holds the Walter G. Ross Chair in Ophthalmic Research.

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