Matrilysin Cleavage of Corneal Collagen Type XVIII NC1 Domain and Generation of a 28-kDa Fragment

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PURPOSE. To localize endostatin and collagen type XVIII in human corneas and to characterize the enzymatic action of matrix metalloproteinases (MMPs) in the cleavage of collagen type XVIII and generation of endostatin in the cornea.

METHODS. Anti-endostatin and anti-hinge antibodies were generated using peptide fragments corresponding to the endostatin region and the adjacent nonendostatin hinge region of collagen XVIII noncollagenous (NC)1 domain, respectively. Confocal immunostaining was performed to localize collagen XVIII in human corneas. SV40-immortalized corneal epithelial cells were immunoprecipitated and incubated with active MMP-1, -2, -3, -7, or -9, and Western blot analysis was performed to study collagen XVIII cleavage. Incubation with MMP-7 was performed at various concentrations (0, 2, 4, and 6 μg/ml) and time intervals (0, 1, 5, and 12 hours). Purified recombinant NC1 fragment of collagen XVIII was also digested with MMP-7, and the cleavage product was sequenced.

RESULTS. Collagen XVIII was immunolocalized to the human corneal epithelium, epithelial basement membrane, and Descemet membrane. Western blot analysis demonstrated a 180- to 200-kDa band corresponding to collagen XVIII. MMP-7 (but not MMP-1, -2, -3, -7, or -9) cleaved corneal epithelium–derived collagen XVIII to generate a 28-kDa endostatin-spanning fragment in a time- and concentration-dependent fashion. MMP-7 cleaved purified recombinant 34-kDa NC1 fragment of collagen XVIII in the hinge region to generate a 28-kDa fragment.

CONCLUSIONS. Collagen XVIII is present in human cornea. MMP-7 cleaves the collagen XVIII NC1 domain to generate a 28-kDa fragment in the cornea. (Invest Ophthalmol Vis Sci. 2001;42:2517–2524)

Collagen type XVIII is a nonfibrillar collagen belonging to the heparan sulfate proteoglycan family.1,2 It is composed of 10 collagen domains alternating with 11 noncollagenous (NC) domains. The N terminus (NC11) and C terminus (NC1) noncollagenous domains have a non–triple-helical structure.3 The NC1 region contains three functionally distinct regions: an association domain (necessary for collagen XVIII oligomerization), a hinge domain (sensitive to proteases cleavage), and the endostatin domain (a 20-kDa fragment with potent anti-angiogenic properties).4 Collagen XVIII is mainly localized to the vascular and epithelial basement membrane.5,6 Several isoforms of collagen XVIII cDNAs have been isolated. In the mouse, three isoforms (two in humans) have been shown to be differentially expressed among tissues.7 In humans, the shorter isoform is expressed in the heart, kidney, placenta, ovaries, skeletal muscle, and small intestine, whereas the longer variant is liver specific. In the eye, collagen XVIII localization has been reported in the retina (inner limiting membrane and pigment epithelium) and the lens capsule but has not been reported in the cornea.8

Cleavage of collagen types IV, XV, and XVIII by proteases generates fragments possessing anti-angiogenic properties (arresten, restin, and endostatin, respectively).9–11 Several proteases, including cathepsin L12 and elastase,13 have been demonstrated to be involved in the cleavage of the C-terminal noncollagenous domain (NC1) of collagen type XVIII to produce endostatin.

Matrix metalloproteinases (MMPs) and other proteases may also be involved in the cleavage of collagen XVIII.12 The MMP family encompasses 24 members that share a calcium and zinc dependency and a high rate of homology in the catalytic domain.13–15 MMPs are involved in extracellular matrix (ECM) remodeling, wound healing, development, cancer invasion, and angiogenesis.17 Several MMPs (types 1, 2, 7, 8, 9, and 13) are known to degrade native collagens (types I, II, III, IV, and VI) and gelatin.18 However, cleavage of collagen XVIII by MMPs and the proteolytic release of endostatin have not been fully characterized.

We previously reported the expression of MMPs in the cornea under normal (MMP-2 and -7) and wound-healing conditions (MMP-9, -13, and -14).19–22 The purpose of this study is to investigate whether collagen XVIII is present in the cornea and to identify the MMPs that are capable of degrading corneal collagen XVIII to generate endostatin-like fragments.

MATERIALS AND METHODS

Cell Culture and Tissue

The SV40-immortalized human corneal epithelial cell line was a gift from Hiroshi Handa, Kyoto Institute of Technology, Kyoto, Japan.23 Corneal epithelial cells were grown to confluence, scraped, and lysed with extraction buffer containing 8 M urea, 50 mM Tris-HCl (pH 8.5), 150 mM NaCl, 1% NP-40 and protease inhibitor combination (Sigma Chemical Co., St. Louis, MO).

Human corneal specimens were obtained from donor corneal tissue, embedded in optimal cutting temperature (OCT) compound (Miles, Elkhart, IN), and flash frozen. Eight-micrometer sections were processed for immunohistochemistry.
Generation and Characterization of Anti-Collagen XVIII Antibodies

The study was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Female New Zealand white rabbits were immunized using synthetic peptides containing residues of the murine 38-kDa NC1 domain (Research Genetics Inc., Huntsville, AL). The first peptide, DDILANPPRLPDRQPYPGVPHH, contained 23 residues in the N-terminal of endostatin domain of NC1 (Fig. 1). Rabbit anti-mouse polyclonal antibodies to the hinge and endostatin domains were generated. They were affinity purified and used for immunohistochemical and Western blotting experiments.

Collagen XVIII immunocomplexes were prepared by incubating mouse liver lysate with anti-collagen antibodies (anti-endostatin or anti-hinge), and Western blot analysis was performed.

Confocal Immunohistochemistry

Cryosections (8 μm) of human corneas were mounted on glass slides (Superfrosted/Plus; Fisher Scientific, Pittsburgh, PA) and kept at room temperature for 30 minutes. A blocking solution (1% BSA in PBS) was applied for 30 minutes at room temperature, followed by incubation with primary antibodies, either anti-hinge, anti-endostatin or type IV collagen (Southern Biotechnology Associates, Inc., Birmingham, AL) of 1:100 dilution for 60 minutes. Samples were then washed with PBS, and the secondary antibody, either rhodamine-conjugated donkey anti-goat of 1:400 dilution or FITC-conjugated affinity purified donkey anti-rabbit IgG of 1:100 dilution (Jackson ImmunoResearch Laboratories, West Grove, PA), was applied for 30 minutes. After the PBS wash, the specimens were mounted with anti-fading medium (Vectashield; Vector Laboratories, Burlingame, CA). The sections were examined with a confocal microscope (TCS4D; Leica, Heidelberg, Germany). Negative control samples (without primary antibody) were similarly processed using the same procedure.

Western Blot Analysis

Immunoprecipitate of collagen XVIII (using anti-hinge antibody) were run on 10% to 20% pre-cast SDS polyacrylamide gels (Novex, San Diego, CA), then electrotransferred onto membranes (Immobilon P, Millipore, Bedford, MA). Membranes were blotted with anti-endostatin antibodies and reacted with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG secondary antibodies (New England Nuclear, Boston, MA). After a washing with TBST for 15 minutes three times, immunoblots were developed with enhanced chemiluminescence (ECL) reagent (Western blot detection reagents; NEN-Life Science Products, Boston, MA). To test the reproducibility of our data, we used two additional anti-collagen XVIII antibodies (gift from Bjorn Olsen, Harvard Medical School, Boston, MA): anti-NC1 antibody (against the...
C-terminal end of collagen XVIII which includes the endostatin domain); and anti-NC11 (against the N-terminal end of collagen XVIII) for immunoprecipitation or immunoblotting, respectively.

Western blots were performed under nonreducing or reducing (achieved with β-mercaptoethanol) conditions. Because the collagen XVIII band is masked by the IgG complex under nonreducing conditions, incubation with MMPs was performed under nonreducing conditions. Immunoprecipitation of cultured human corneal epithelial cell lysate was performed using anti-hinge antibodies and immunoblot analysis was performed with anti-endostatin antibody. Immunoprecipitates were incubated without MMPs (lane 1) and with MMPs-7, -2, and -9 (lanes 2, 3, and 4, respectively). MMP-7 incubation (2 μg/ml) for 1 hour showed evidence of cleavage of collagen XVIII to generate a 28-kDa fragment (lane 2). Incubation with MMP-2 and -9 did not generate a 28-kDa fragment (lanes 3, 4). Immunoprecipitates incubated with active MMP-1, -2, -3, and -9 (with and without an additional step of APMA activation) revealed no additional change of collagen XVIII with the additional step of APMA activation.
tions and the endostatin band hidden by IgG light chain under reducing conditions, the two methods were performed depending on the band of interest.

Cleavage of Collagen XVIII by MMPs
Collagen XVIII immunocomplexes were prepared by incubating cell lysates with anti-hinge antibodies. The immunocomplexes were then washed twice with washing buffer and once with phosphate-buffered saline and then incubated with active MMP-1, -2, -3, -7, or -9 enzyme in substrate buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 50 mM ZnSO₄) for 1 hour at 37°C. MMP-1 and -3 enzymes were obtained from Sigma Chemical Co. and MMP-2, -7, and -9 from Calbiochem (San Diego, CA). Aminophenylmercuric acetate (APMA; 1 mM at 37°C for 2 hours; Sigma) was used to activate inactive enzymes. Combinations of MMPs (MMP-1 and -7, MMP-2 and -7, MMP-3 and -7, MMP-9 and -7, MMP-1 and -3, or MMP-3 and -9) were also used. Various MMP-7 concentrations (0, 2, 4, and 6 μg/ml) and incubation times (0, 1, 5, and 12 hours) were used. The reaction was stopped by adding 2 × SDS gel loading buffer and boiling for 2 minutes, and Western blot analysis was performed as described earlier.

RESULTS
Western Blot Analysis with Anti-endostatin and Anti-hinge Antibodies
Western blot analysis of corneal epithelial cells (with and without immunoprecipitation with anti-endostatin and anti-hinge antibodies) under reducing conditions revealed a 180- to 200-kDa band corresponding to collagen XVIII (Fig. 2A). Immunoprecipitation showed an IgG band at the 50-kDa position (Fig. 2A, lanes 2 and 4). Under nonreducing conditions (Fig. 2B, lane 6) the IgG band was centered at the 150-kDa position, which masked the collagen XVIII immunoreactive band. To confirm the specificity of our antibodies (anti-hinge and anti-endostatin antibodies), liver lysates were used for immunoprecipitation and further analyzed by Western blot analysis. As shown in Figure 2C under reducing conditions, characteristic high-molecular-weight bands were seen at the 180- to 240-kDa position. (These multiple reactive bands of collagen XVIII may be due to alternative splicing and/or heparan sulfate modification in the liver.)

Immunohistochemistry of Collagen XVIII in Human Cornea
Confocal immunostaining with anti-hinge and anti-endostatin antibodies revealed collagen XVIII immunolocalization to the corneal epithelium and epithelial basement membrane (Fig. 3A) and to the Descemet membrane (Fig. 3D). Epithelial staining was uniform in all layers. No differences between collagen XVIII immunolocalization were observed between anti-hinge (Fig. 3A) and anti-endostatin (Fig. 3G) antibody staining. Colocalization of collagen XVIII and type IV collagen was observed in the epithelial basement membrane (Fig. 3C) and the stromal side of the Descemet membrane (Fig. 3F). No staining was seen in the negative control specimens (Fig. 3H).

Collagen XVIII Cleavage by MMPs
For collagen XVIII cleavage experiments, nonreducing conditions were used. To characterize the potential role of matrix metalloproteinases in the generation of endostatin, collagen

![Figure 5](https://example.com/image5.png)

**Figure 5.** Comparison of MMP-7–treated immunoprecipitates of cultured human epithelial cell lysates with lysate-free samples using Western blot analysis (A) and silver staining (B) (performed under nonreducing conditions). Lane 1: no lysate or immunoprecipitation (lysis buffer and protein A incubated for 1 hour at 37°C); lane 2: addition of MMP-7 active enzyme to sample in lane 1; lane 3: no lysate and addition of anti-hinge antibody incubated for 1 hour; lane 4: MMP-7 incubated with anti-hinge antibody; lane 5: epithelial cell lysate immunoprecipitated with anti-hinge antibody; lane 6: incubation with MMP-7 showing a 28-kDa breakdown product that stains with anti-endostatin antibody (Arrow; A). Silver staining (B) was performed on the identical samples as indicated in (A). Lanes 2, 4, and 6: addition of MMP-7 active enzyme showed a 19-kDa band that did not stain with anti-endostatin antibody. (A: lane 6, arrow) The presence of the 28-kDa band suggests that this fragment is generated by MMP-7–mediated cleavage of collagen XVIII.
XVIII was immunoprecipitated from cultured human corneal epithelial cells and incubated with active MMP-2, -7, and -9 for 1 hour. Western blot analysis was then performed. As shown in Figure 4A, collagen XVIII can be cleaved by active MMP-7 to generate a 28-kDa fragment. MMP-1, -2, -3, and -9 (lanes 2-4, 6) did not cleave collagen XVIII. (B, C) With increasing time of incubation (B) and with increasing MMP-7 concentrations (C), the 28-kDa band showed corresponding increases in density. (D) Western blot analysis showing cleavage of collagen XVIII by combination of MMPs. MMP-1, -2, -3, and -9 did not provide an apparent synergistic effect with MMP-7.

To eliminate the possibility that the 28-kDa matrilysin-derived endostatin-like fragment came from either immunoprecipitated antibodies, active MMP-7, or protein A, Western blot analysis was performed with and without lysate (Fig. 5A). A 28-kDa fragment was generated only in the presence of lysate, anti-hinge antibody, and active MMP-7 (Figs. 5A; lane 6), suggesting that the origin of this band was collagen XVIII. This 28-kDa band was not seen on silver staining under similar conditions (Fig. 5B), which may imply a low amount of generated fragment.

**Figure 6.** Immunoprecipitation experiments with human corneal epithelial cell lysate, using anti-NC11 antibodies. Western blot analysis with anti-NC1 antibodies was performed under nonreducing conditions to determine the cleavage of collagen XVIII immunoprecipitated by MMPs. (A) MMP-7 (lane 5) degraded collagen XVIII to generate a 28-kDa fragment. MMP-1, -2, -3, and -9 (lanes 2-4, 6) did not cleave collagen XVIII. (B, C) With increasing time of incubation (B) and with increasing MMP-7 concentrations (C), the 28-kDa band showed corresponding increases in density. (D) Western blot analysis showing cleavage of collagen XVIII by combination of MMPs. MMP-1, -2, -3, and -9 did not provide an apparent synergistic effect with MMP-7.
To confirm the cleavage of collagen XVIII by MMPs, two additional anti-collagen XVIII antibodies (anti-NC1 and anti-NC11) were used (Fig. 6). Incubation with active MMP-7 generated a similar 28-kDa band, but not with MMP-1, -2, -3, or -9 (Fig. 6A). MMP-7 generated this 28-kDa fragment in a time- and concentration-dependent fashion (Figs. 6B, 6C).

To address whether a combination of MMPs is required for collagen XVIII processing, a mixture of MMPs was used to assay the processing of immunoprecipitated collagen XVIII (Fig. 6D). We found that the addition of other MMPs (types 1, 2, 3, and 9) to MMP-7 did not alter the cleavage pattern of collagen XVIII. In addition, combinations of MMP-1 and -3 and MMP-3 and -9 did not generate additional fragments of collagen XVIII (Fig. 6D).

Cleavage and Sequencing of Recombinant Collagen XVIII NC1 Domain with MMP-7

As shown in Figure 7A, a 28-kDa endostatin-spanning molecule was produced by the cleavage of a 34-kDa NC1 fragment by active MMP-7 enzyme. The 28-kDa fragment was subjected to N-terminal protein sequencing and was shown to have an N-terminal amino acid sequence of LxDSNPYPRR which are located in the hinge region of NC1 domain (Fig. 7B). Thus the cleavage site of MMP-7 differs from that of MMP-9, elastase, and cathepsin L (Fig. 7B).

DISCUSSION

In this study, collagen XVIII was localized in the corneal epithelium, epithelial basement membrane, and Descemet membrane. We believe that this is the first published report demonstrating the presence of collagen XVIII in the human cornea. In addition, we demonstrated matrilysin-induced proteolysis of collagen XVIII to release a 28-kDa fragment and characterized the cleavage site in the hinge region of NC1 domain.

Ingber and Folkman postulated that collagen metabolism may play a role in regulating angiogenesis. This is consistent with studies showing the anti-angiogenic activity of noncollagenous domain proteolytic fragments of collagen types XVIII (endostatin) and XV (restin) and type IV collagen chains α1 (arrestin), α2 (canstatin), and α3 (tumstatin). Among proteases, the matrix metalloproteinases are of special interest in their ability to cleave ECM molecules and cell surface proteins. MMPs regulate the assembly of the ECM and the release of bioactive fragments and growth factors during growth, morphogenesis, tissue repair, and pathologic processes. These enzymes are distinguished from other classes of proteinases by their dependence on metal ions and a neutral pH to be active. There is evidence that MMPs generate angiostatin from plasminogen.
to generate a 28-kDa fragment. This fragment differs from the published endostatin molecule, which is a 20- to 22-kDa fragment with antiangiogenic properties. We have not as yet ascertained the antiangiogenic potential of this 28-kDa matrix-derived fragment. Human MMP-7 is known to cleave ECM and basement membrane proteins, such as fibronectin, collagen type IV, laminin, elastin, entactin, and proteoglycan aggregates. MMP-7 also mediates proteolytic processing of the precursor of tumor necrosis factor α and urokinase plasminogen activator and has been shown to release membrane-bound Fas ligand. It has also been shown to cleave plasminogen and generate angiostatin, another potent antiangiogenic molecule. We have reported the presence of MMP-7 in the cornea and its upregulation after excimer laser keratectomy. We localized MMP-7 in the epithelium and the basement membrane area, suggesting a colocalization of this enzyme with collagen XVIII, which belongs to the heparan-sulfate proteoglycan family of molecules, known to act as MMP-7 ECM docking molecules.

Because MMP-7 is the smallest member of the MMP family, it may easily access the collagen XVIII cleavage site. Ferreras et al. have recently reported that the recombinant collagen XVIII NC1 domain can be processed by MMP-3, -9, -12, and -13 with a relative low activity. In the recombinant NC1 fragment, cleavage sites may be more accessible to MMPs than immunoprecipitated native collagen molecules. The Western blot experiments used in our study suggest that, under our experimental conditions (exposure and concentration), MMP-7 is more efficient than the other tested MMPs in generating a 28-kDa molecule, but our experiments do not establish whether MMP-7 plays a role in the cleavage of corneal collagen XVIII in vivo. Further investigations may allow us to assess the biological function of this 28-kDa fragment generated by the enzymatic activity of MMP-7.

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