MMPs and Proteinase Inhibitors in the Human Aqueous Humor

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Purpose. This study was performed to examine the gelatinolytic and caseinolytic activities and the levels of two proteinase inhibitors, α1-proteinase inhibitor (α1-antitrypsin) and α2-macroglobulin, in the human aqueous humor.

Methods. Aqueous humor samples were collected during elective surgery in patients with cataracts. Zymography with gelatin- and casein-containing gels was performed. The inhibitors were examined by Western blot analyses, enzyme-linked immunosorbent assay, and dot blot assays.

Results. The aqueous humor contained a major band of gelatinolytic activity at a molecular weight of 66 kD and minor bands at 125, 95, and 62 kD. These gelatinases were inhibited by 10 mM ethylenediaminetetraacetic acid (EDTA) or 1,10-phenanthroline. After extended incubation (48 hours), zymography on casein-containing gels showed proteinase bands with molecular weights in the 80- to 84-kD range. Additional bands at 68 and 48 kD also were observed. All the caseinase activities were inhibited by 10 mM phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin. No inhibition was observed with 5 mM EDTA, 5 μM E-64, or 1 nM pepstatin. These results indicated that the caseinases are serine proteinases. Western blot analysis showed a 53-kD α1-proteinase inhibitor band in the aqueous humor. The concentration was 32.2 ± 9.9 μg/ml, constituting approximately 15% of the total protein. A 360-kD protein band immunoreactive to anti-α2-macroglobulin also was detected. Its level in the aqueous humor was 3.2 ± 1.3 μg/ml.

Conclusions. The gelatinases, serine-like proteinases, and proteinase inhibitors found in the aqueous humor may participate in the remodeling of extracellular matrices in the trabecular meshwork and other tissues bordering the anterior chamber. Invest Ophthalmol Vis Sci. 1993;34:3541-3548.

Recent studies have shown the roles of a class of enzymes known as matrix metalloproteinases (MMPs) in connective tissue degradation and remodeling. The MMPs have been grouped into three subfamilies—collagenase, gelatinase, and stromelysin—based on the specificity for different extracellular matrix components. For instance, the interstitial collagenase, designated as MMP-1, degrades native collagen types I, II, and III. Stromelysin, MMP-3, cleaves proteoglycans, fibronectin, laminin, gelatins, and casein, whereas the 72-kD gelatinase, MMP-2, and the 92-kD gelatinase, MMP-9, show activities against gelatins of all types and native collagen types IV, V, and VII. The MMP family members share considerable sequence similarity, and the different proteinases appear to have evolved from a common ancestral gene. These enzymes are secreted as proenzymes and are activated by cleavage of a polypeptide from the amino terminal portion of the molecule. They have been implicated in normal remodeling processes, such as those involved in developmental events and wound healing. Abnormal expression of MMPs has been associated with pathologic conditions including rheumatoid arthritis, corneal ulcer-
of the anterior chamber. The aqueous humor provides nutrition to the surrounding avascular tissues, including the cornea, lens, and trabecular meshwork, and functions as a reservoir, accommodating metabolites from the various types of cells. The physical and chemical properties of the aqueous humor have been thought to play an important role in influencing the dynamics of its outflow and the development of pathologic conditions. Investigators have detected many components of normal serum, including transferrin, transforming growth factor-β, and fibronectin, in the aqueous humor. In addition, type I collagenolytic activity and tissue-type and urokinase-type plasminogen activator have also been found in the aqueous humor.

In view of the importance of the MMPs and other proteinases, we sought to determine whether the human aqueous humor contains these molecules. In addition, we extended previous investigations by examining the levels of α1-proteinase inhibitor and α2-macroglobulin in the aqueous humor.

MATERIALS AND METHODS

Aqueous Humor Sampling

Human aqueous humor was collected from 11 patients undergoing elective surgery for senile cataract by a previously described method. Their ages ranged from 60 to 84 years (mean ± SD, 69.5 ± 6.5 years). The patients had no ocular diseases other than cataract. In addition, none of them had general diseases such as type II diabetes mellitus or hypertension. Approximately 100 to 130 μl aqueous humor was obtained from each subject. The collected samples were stored at −20°C and thawed just before the experiments.

The tenets of the Declaration of Helsinki were followed. Informed consent was obtained from all patients, and approval was granted by the institutional human experimentation committee.

Protein Determination

The protein concentration in the sample was determined by the method of Bradford with bovine serum albumin as the standard. Aliquots of the human aqueous humor (2 μl) were pipetted into flat-bottom 96-well plates (Costar, Cambridge, MA), and Coomassie brilliant blue G-250 dye reagent (Bio-Rad, Richmond, CA) was added to each well. The optical density of each well was measured at 600 nm with a microplate reader (Biotek, Burlington, VT). The protein content of the aqueous humor samples was determined from the standard albumin curve.

Zymography

Zymography was performed as previously described in sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis with gels containing substrates, such as gelatin (for gelatinase) and casein (for stromelysin and serine proteinases), to analyze the content of specific proteinases in the aqueous humor samples. In brief, 10% or 11% SDS–polyacrylamide gels were prepared, and 1.5 mg/ml of either bovine skin gelatin (type A, Sigma Chemical Company, St. Louis, MO) or casein (Nutritional Biochemicals, Cleveland, OH) was copolymerized in the gel. Human aqueous humor samples (12.5 to 20 μl each) were mixed with an equal volume of 2X sample buffer, loaded onto the gel without boiling, and electrophoresed at 4°C under nonreducing conditions. The gel then was shaken for 1 hour in a 2.5% solution of Triton X-100 (Sigma) at room temperature to remove the SDS and incubated at 37°C in a reaction buffer (50 mM Tris, pH 7.5; 10 mM CaCl₂) overnight to allow proteinase digestion of the substrates. Some casein gels also were incubated for 48 hours in 50 mM Tris (pH 8.0), 5 mM CaCl₂, and 0.02% azide. After staining with Coomassie brilliant blue R-250 (Bio-Rad), the positions of enzymatic activities were identified as clear bands in the stained background. Molecular weight standards (Bio-Rad) and trypsin (as a positive control; Sigma) were run in parallel.

In some gelatinase experiments, one of the metalloproteinase inhibitors, ethylenediaminetetraacetic acid (EDTA) (dissolved in water; Sigma) or 1,10-phenanthroline (dissolved in methanol; Sigma), was included in the 2.5% Triton X-100 solution for SDS/Triton exchange, as well as in the Tris–CaCl₂ buffer dur-
ing proteinase reactions. The final concentration of each inhibitor was 10 mM.

To further examine the caseinolytic enzymes, inhibitors were added to a final concentration of 5 mM for EDTA, 10 mM for phenylmethylsulfonyl fluoride (dissolved in isopropanol; Sigma), 1 µg/ml for aprotinin (dissolved in water; Sigma), 5 µM for E-64 (L-trans-epoxy succinyl-leucylamido(4-guanidino)-butane or N-[N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]agmatine, dissolved in water; Sigma), or 1 µM for pepstatin (dissolved in methanol; Sigma) to the samples before electrophoresis and to the gel incubation solutions used after electrophoresis.

**Sodium Dodecyl Sulfate—Polyacrylamide Gel Electrophoresis and Immunodetection of Proteinase Inhibitors**

Human aqueous humor samples (20 µl) were electrophoresed on 5% to 20% gradient gels under reduced conditions. The proteins then were electrophoblated overnight onto a nitrocellulose membrane. After blocking with 5% Carnation nonfat dry milk, the membrane was incubated with rabbit anti-α1-proteinase inhibitor (1:1000; DAKO, Santa Barbara, CA) and biotinylated goat anti-rabbit IgG. Immunodetection was performed with the Vectastain ABC system (Vector Laboratories, Burlingame, CA) with 3,3-diaminobenzidine (Sigma) to develop the color. Prestained molecular weight markers (Bio-Rad) and normal human serum samples were run in parallel.

Human aqueous humor samples (12.5 µl) and α2-macroglobulin (Athens Research Technology, Athens, GA) were electrophoresed on 6% SDS gels under nonreducing conditions. The gels were electrophoblated, and the nitrocellulose membranes were blocked and washed in the same manner as those for α1-proteinase inhibitor. The blots were allowed to react with either rabbit anti-α2-macroglobulin (1:2000; Accurate Corporation, Westbury, NY) or rabbit IgG (1:2000; Sigma), and then they were incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:2000; Bio-Rad). Immunoreactive bands were visualized with the luminol-based ECL system (Amersham, Arlington Heights, IL) or rabbit peroxidase-labeled goat anti-rabbit IgG. The dots were visualized by the ECL system and quantified with a microplate reader.

**RESULTS**

The total protein concentration in the aqueous humor samples was 151 to 286 µg/ml (mean ± SD, 218.1 ± 54.2 µg/ml). The electrophoretic pattern of proteins by Coomassie blue staining was similar in all samples (photographs not shown).

**Enzymes in the Aqueous Humor**

Zymography of gelatin-containing gels showed a major gelatinase band at a molecular weight of 66 kD (Fig. 1). Minor bands were also apparent at molecular weights of 125, 95, and 62 kD in the human aqueous humor (Fig. 1). Trypsin, run as a positive control, showed bands at 29 and 24 kD. The gelatinase activities were inhibited by treatment with 10 mM of metal chelators EDTA (Fig. 2) and 1,10-phenanthroline, indicating that all these bands are products of metallo-proteinase activities.

The gel containing casein that was incubated overnight showed only the bands of trypsin (Fig. 3). When the casein-containing gels were incubated for 48 hours (Fig. 4), caseinase activities at the molecular weight range of 80 to 84 kD appeared. A band at 68 kD (Figs. 4, 5) and a minor band at 48 kD also were observed (Fig. 4).

For additional examination of the caseinases present, the aqueous humor samples and gels were incubated in the presence of various proteinase inhibitors. The caseinolytic activities seen in the untreated gel
FIGURE 1. Zymography of gelatinolytic activities in the human aqueous humor. Aqueous samples (20 µl) were electrophoresed on a gelatin-containing 11% gel. One major band at 66 kD and minor bands at 125, 95, and 62 kD were seen in eight human aqueous samples from patients with cataracts (lanes 1 to 6, 8, and 9). Trypsin (0.5 µg, lane 7) showed major bands at 29 and 24 kD.

FIGURE 2. Zymography of gelatinolytic activities in the human aqueous humor in the presence of EDTA. Gelatinase activities in the eight human aqueous samples (lanes 1 to 8) used for Figure 1 were inhibited by pretreatment of 10 mM EDTA and inclusion of 10 mM EDTA during incubation of gels in 2.5% Triton X-100, as well as in the Tris-CaCl₂ buffer. The activity of the major trypsin band (0.5 µg, lane 9) seen at 24 kD and that of the minor band at 29 kD also was inhibited significantly by EDTA.

FIGURE 3. Zymography of caseinolytic activities in the human aqueous humor. Aqueous samples (20 µl) were electrophoresed on a casein-containing 11% gel and incubated for 18 hours in the Tris-CaCl₂ buffer. The major trypsin bands (0.5 µg, lane 1) were seen at 31 and 24 kD. No activity was visible at approximately 52 kD, where stromelysin is expected in eight human aqueous samples from patients with cataracts (lanes 2 to 9).

FIGURE 4. Zymography of caseinolytic activities in the human aqueous humor. Five aqueous samples (12.5 µl each) from patients with cataracts were electrophoresed on a casein-containing 10% gel and incubated for 48 hours in 50 mM Tris (pH 8.0), 5 mM CaCl₂, and 0.02% azide. (a) The position of the 84- to 80-kD protein bands. (b) The position of the 68-kD band. (c) The position of the 48-kD band.
Proteinases and Inhibitors in the Aqueous Humor

**Proteinase Inhibitors in Aqueous Humor**

A major 53-kD protein band corresponding to α1-proteinase inhibitor molecule (Fig. 6) was seen with Western blot immunodetection in all aqueous humor samples and serum. With the enzyme-linked immunosorbent assay, the aqueous humor concentration of α1-proteinase inhibitor was found to be 32.2 ± 9.9 μg/ml (range, 16.0 to 50.4 μg/ml; n = 11). This amounted to 14.9 ± 4.5% of the total protein in human aqueous humor.

α2-Macroglobulin was detected on Western blots when the SDS gels were run under nonreducing conditions as a band co-migrated with the purified serum α2-macroglobulin (Fig. 7). The calculated molecular weight was 360 kD. The level of α2-macroglobulin in the human aqueous humor determined by a dot blot assay was 3.2 ± 1.3 μg/ml (range, 1.3 to 4.6 μg/ml; n = 6).

**DISCUSSION**

All aqueous humor samples used were collected during elective surgery in patients with cataracts because previous studies have verified that the postmortem aqueous humor is not a reliable source of samples for analyses of components in vivo. The protein concentration in our samples was well within the range of those reported in the literature (98 to 360 μg/ml).29,34-36

The abundant 66-kD gelatinase found by zymography is similar in molecular size to that extracted from the normal corneal stroma and trabecular meshwork cultures. This enzyme emerged as a 72-kD protein band on reduction and Western blot analysis and was identified as the proenzyme form of 72-kD gelatinase MMP-2. The 95-kD gelatinase is in the size range to be the proenzyme form of MMP-9, a 92- to 95-kD gelatinase produced by cultures of corneal epithelium and corneal fibroblasts and by trabecular meshwork cultures after phorbol ester induction. There is also another possibility that the lower molecular weight bands in the aqueous humor could represent breakdown products of the 125-kD band, which might be related to the 130-kD species found in the plasma.

The gelatinases in the aqueous humor are likely to be contributed in part by the cornea and other surrounding cells and tissues; however, most of the aqueous humor gelatinases still may be derived from the human plasma, in which gelatinases with molecular weights of 92, 66, and 62 kD (besides the 130-kD gelatinase) were detected.

Two major bands at 29 and 24 kD were observed for the trypsin used. The higher molecular weight band could be a contaminating protease or another form of the enzyme that is folded differently, migrating more slowly than the 24-kD form. This latter alternative is possible because the sample was not boiled before electrophoresis, nor was a reducing agent added.
FIGURE 6. Western blot analysis of α1-proteinase inhibitor. The α1-proteinase inhibitor standard (500 ng, lane 1), normal human serum (5 μg protein, lane 2), and five human aqueous humor samples from patients with cataracts (lanes 3 to 7) all showed a major protein band of 55 kD immunoreactive to an anti-α1-proteinase inhibitor antibody.

In agreement with a previous article,25 proteinase bands in human aqueous humor samples were not found in casein-containing gels when incubated overnight. Caseinolytic activities, however, were shown after extended incubation of casein-containing gels. This suggests that the caseinases are not present in appreciable amounts. The caseinases observed were inhibited by the serine proteinase inhibitors aprotinin and phenylmethylsulfonyl fluoride, but not by the cysteine proteinase inhibitor E-64, aspartic proteinase inhibitor pepstatin, or metalloproteinase inhibitor EDTA. These inhibitor results indicate that the caseinases are serine proteinases. The enhancement of the 68-kD proteinase band by EDTA, methanol, and isopropanol suggests that this enzyme is more active after a cation is removed or when a conformational change occurs.

α1-Proteinase inhibitor was shown to represent approximately 15% of the total protein in the human aqueous humor. The reported value of this inhibitor in the human serum was 2.5 ± 0.4 mg/ml,41 constituting approximately 3% of the total serum protein. The greater proportion of α1-proteinase inhibitor relative to total protein in the aqueous humor compared with serum suggests that, in addition to filtration from the serum, this inhibitor may be degraded to a lesser degree in the aqueous humor, secreted preferentially by the ciliary processes, or actively produced locally by corneal and other types of cells. The latter possibility is supported by previous studies in which this inhibitor was localized in the cornea33 and trabecular meshwork.42

Western blot analysis of the aqueous humor indicated the presence of a 360-kD dimer form of α2-macroglobulin held together by disulfide bonds.43,44 It is unknown whether the α2-macroglobulin molecule actually exists as a dimer (360 kD) or tetramer (720 kD) because the ionic bonds between disulfide-bridged dimers are broken in the presence of SDS. Both forms of the inhibitor are active,43,44 with the tetramer being the form found in serum. The α2-macroglobulin level in the aqueous humor is approximately 1000-fold less than that in serum.45 This level was still greater than that expected because filtration of high molecular weight proteins from the serum is not favored; however, the inhibitor may be synthesized and secreted by tissues surrounding the anterior chamber. Corneal cells, for example, have been shown to contain46 and produce47 α2-macroglobulin.

The gelatinases, serine-like proteinases, and proteinase inhibitors in the aqueous humor may partici-
in the remodeling of extracellular matrices in the trabecular meshwork and in other tissues bordering the anterior chamber. The level and balance between the enzymatic action and inhibitory defense conceivably are essential in the turnover of tissue constituents and the normal physiology. Activity changes in the enzyme or inhibitor may upset the balance, resulting in excessive or insufficient degradation processes, and, in turn, they may affect the structure and integrity of cells and tissues. It is also noteworthy that α-proteinase inhibitor, which forms a 1:1 complex with its target enzymes, such as elastase, cathepsin G, and plasmin, may be cleaved and inactivated by metalloproteinases, including gelatinases. Our data suggest that gelatinase in the aqueous humor under normal conditions is mostly the inactive 66-kD proenzyme form of MMP-2. Under conditions such as inflammation, the proenzyme may be activated, rendering the inhibitor inactivated. These scenarios may trigger uncontrolled proteolysis, leading to tissue damage and serious pathologic consequences.

**Key Words**

aqueous humor, gelatinase, caseinase, zymography, proteinase inhibitor

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