Gelatinase B/matrix metalloproteinase-9 provokes cataract by cleaving lens βB1 crystallin

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ABSTRACT **Cataract is a common cause of blindness and results from destruction of the microarchitecture of the lens. It is observed in many genetic syndromes, infections, inflammatory diseases and during aging. Fluctuations in lens density and light scattering by altered refraction index form the physical basis for this process, but the pathogenesis is poorly understood. Increased levels of gelatinase B/matrix metalloproteinase-9 have been reported for cataract-associated disorders such as eye inflammation and diabetes. We demonstrate that incubation of lenses with gelatinase B leads immediately to cataract. In complete eye extracts, βB1 crystallin was identified as the major gelatinase B substrate by combination of proteomics, mass spectrometry, and Edman degradation analysis. The cleavage of βB1 crystallin was also observed in vivo after endogenous gelatinase B-induction by the chemokine granulocyte chemotactic protein-2 in wild-type mice but not in gelatinase B$^{-/-}$ mice.—Descamps, F. J., Martens, E., Proost, P., Starckx, S., Van den Steen, P. E., Van Damme, J., Opdenakker, G. Gelatinase B/matrix metalloproteinase-9 provokes cataract by cleaving lens βB1 crystallin. *FASEB J.* 19, 29–35 (2004)**

Key Words: neutrophil · crystallins · granulocyte-chemotactic protein-2

THE EYE is architecturally and biochemically organized to maintain high-quality vision. Par excellence, the lens forms a specialized tissue composed of epithelial cells, which form a monolayer across the surface of the tissue, and fiber cells, which constitute the remainder and majority of the tissue volume. Fiber cells are formed continuously by differentiation of epithelial cells, which involves elongation of the cells, withdrawal from the cell cycle, and specialization for the synthesis of crystallin proteins. The lens focuses optical images onto the light-sensitive rods and cones of the highly vascularized retina. The blood-retinal barrier makes the eye an immunoprivileged organ. However, in many disease processes, including uveitis and diabetic retinopathy, the blood-retinal barrier may be compromised and, once it is broken, inflammatory effector cells and molecules cause complications like macular edema, autoimmunity, or irreversible damage like cataract formation (1, 2). Gelatinase B or matrix metalloproteinase-9 (MMP-9) is released by many cells, including leukocytes, and has been associated not only with inflammatory conditions but has been pinpointed as a causative effector of autoimmunity in other immunoprivileged tissues (3). Refractive error blindness is a worldwide problem: annually more than 200,000 children develop cataract, and cataract accounts for 50% of blindness worldwide.

So far, no study has demonstrated that gelatinase B has been implicated in or causes cataract (4). We show that gelatinase B penetrates the lens, cleaves lens substrates, and causes cataract. To identify the target molecules of gelatinase B, we studied the susceptibility of complete eye extracts and lens homogenates to proteolysis by MMP-9 and defined βB1 crystallin as the predominant substrate. To demonstrate the specificity of this enzyme reaction, endogenous gelatinase B was induced in vivo and demonstrated to cleave βB1 crystallin in *MMP9*$^{+/+}$wild-type but not in knockout *MMP-9*$^{-/-}$ mice.

MATERIALS AND METHODS

Reagents

Recombinant human MMP-9 (rhMMP-9) was expressed in insect cells and purified from supernatant using gelatin-Sepharose affinity chromatography. Recombinant mouse granulocyte chemotactic protein-2 (9-78) (GCP-2) was expressed in *Escherichia coli*, purified to homogeneity from the periplasmic fraction in three steps by heparin Sepharose affinity chromatography, cation exchange chromatography, and reversed phase high-performance liquid chromatography (5). Recombinant mouse gelatinase B (rmMMP-9), stromelysin-1 (MMP-3), and tissue inhibitor for matrix metalloproteinases-1 (TIMP-1) were from R&D Systems (Minneapolis, MN, USA); *p*-aminophenylmercuric acetate (APMA) was from Sigma (St. Louis, MO, USA)

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doi: 10.1096/fj.04-1837com

Mouse strains

Wild-type *MMP9*$^{+/+}$ C57BL/6 and knockout *MMP9*$^{-/-}$ mice, backcrossed for 10 generations into a C57BL/6 genetic background, were used (6). All experimental procedures were approved under license LA 1210243 for animal welfare (Project P02082) by the institutional ethics committee.

Incubations with pure gelatinase B

Recombinant human pro-MMP-9 was activated with MMP-3 at a molar ratio of 1:100 (MMP-3: MMP-9) for 1 h at 37°C. Recombinant mouse pro-MMP-9 was activated with 1 mM APMA for 1 h at 37°C. Dissected mouse eyes or lenses were homogenized and sonicated in ice-cold assay buffer (100 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$). Soluble proteins were obtained in the supernatant after centrifugation at 10,000 *g* at 4°C. Protein content was determined with the use of the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Equivalent amounts of lens proteins were incubated with active enzyme for 3 h at 37°C. As a control experiment, a parallel sample was incubated with an equivalent amount of MMP-3 (for activation of pro-MMP-9) under similar conditions. As an additional control, eye extracts were incubated with 10 ng rhMMP-9 in the presence of inhibitors *o*-phenantroline (5 mM) or TIMP-1 in 3-fold molar excess. For ex vivo incubations, intact lenses were incubated in 50 μL minimal essential cell culture medium or assay buffer in the presence of 3 μg active rhMMP-9 or with carrier only.

Preparation of neutrophil cytosolic extract

Human neutrophils from buffy coats were washed three times in 0.9% saline, resuspended in ultrapure water, and subjected to three freeze-thaw cycles. Cell debris was removed by centrifugation (10 min, 20,000 *g*) and the supernatant was collected as the cytosolic fraction. Depletion of MMP-9 was performed using gelatin-Sepharose affinity chromatography. The effectiveness of this method was > 90% as shown by semiquantitative zymography analysis [ref 7 and data not shown]. Before use, MMPs in the extract were activated with 1 mM APMA for 1 h at 37°C.

Ocular injections

To avoid damage to the lens, a volume of 1 μL GCP-2 (in ranging concentrations) in 0.9% saline was carefully injected through the sclera (immediately posterior to the limbus) into the vitreous chamber with the use of a 30-gauge needle connected to a Hamilton syringe. Holding of the needle in the vitreous chamber for 15 s prevented backflow of injected sample after needle withdrawal.

Electrophoresis

Protein digests or lens extracts (30 and 100 μg) were separated by SDS-PAGE using 12% acrylamide gels (29:1 acrylamide: bis-acrylamide) or 2-dimensional gel electrophoresis (2D-GE). Isoelectric focusing was performed on 7 cm, pH 3-7 Immobiline™ Drystrips (Pharmacia, Uppsala, Sweden) using a ZOOM IPG-Runner apparatus (Invitrogen) for 6000 kV-h. Strips were equilibrated for 15 min in 50 mM Tris HCl (pH 6.8), 6 M urea, 30% (v/v/) glycerol, 2% (w/v) SDS, 62 mM DTT, and 0.01% Bromophenol Blue dye, then for 15 min in the same buffer without DTT but containing 150 mM iodoacetamide. Equilibrated strips were loaded onto 12% acrylamide gels and overlaid with 0.5% agarose dissolved in Tris-glycine running buffer. Gels were electrophoresed in Hoefer cells (Pharmacia, Uppsala, Sweden) at 25 mA/gel at room temperature until the dye migrated off the bottom of the gels. All gels were stained with Coomassie Brilliant Blue.

NH_2-terminal amino acid sequencing

For NH_2-terminal amino acid sequencing, proteins were electroblotted on a polyvinylidene difluoride membrane after SDS-PAGE or 2D-GE. After excision from the blots, the proteins were sequenced on a capillary protein sequencer (Procise 491 cLC; Applied Biosystems, Foster City, CA, USA).

Protein identification by mass spectrometry (MS) after in-gel tryptic digest

NH_2-terminally blocked proteins were identified on an Ultraflex (Bruker, Bremen, Germany) matrix-assisted laser desorption ionization time-of-flight/time-of-flight mass spectrometer (MALDI-TOF-TOF MS) after in-gel tryptic digest. Protein spots were manually excised from the gel. Spots were destained with 50 mM NH_4HCO_3/acetonitrile 1:1 (v/v), dehydrated with acetonitrile, and proteins were reduced with 10 mM DTT at 56°C for 45 min. Alkylation was performed with 55 mM iodoacetamide in 25 mM NH_4HCO_3 (30 min, room temperature). The gel pieces were dehydrated again with acetonitrile, rehydrated with 25 mM NH_4HCO_3 containing 5 ng/μL sequencing grade trypsin, and incubated at 37°C overnight. The resulting tryptic peptides were extracted from the gel by sonication in 1% formic acid/50% acetonitrile.

Zymography

Five microliters from a complete mouse eye extract (100 μL) were prepurified using mini gelatin-Sepharose affinity chromatography as described earlier (7). Zymography was performed using 7.5% acrylamide gels with 0.1% copolymerized gelatin.

RESULTS

Identification of βB1 crystallin as the major ocular substrate of gelatinase B

To investigate the potential detrimental effect of gelatinase B in the eye, our goal was to identify the repertoire of gelatinase B substrates in the eye using a so-called degradomics approach (8). We first incubated 30 μg soluble protein preparations from total eye homogenates of a 9-wk-old mouse eye with 10 ng rhMMP-9 for 3 h. Under these limiting enzyme conditions, a 31 kDa protein band was degraded (**Fig. 1*A***), identified as βB1 crystallin by MALDI-TOF/TOF mass spectrometry analysis of an in-gel tryptic digest. Based on the observation that lens crystallins in young mice are more intact than in adult mice, we performed incubations of 30 μg lens proteins from a 2-wk-old mouse (Fig. 1*B*). To determine degradation efficiency, we prepared decreasing concentrations of active recombinant human rhMMP-9 for the assays. βB1 crystallin was degraded after 3 h with only 1 ng active rhMMP-9 in a final volume of 10 μL, i.e., at an estimated enzyme:substrate ratio of 1: 1000. Control

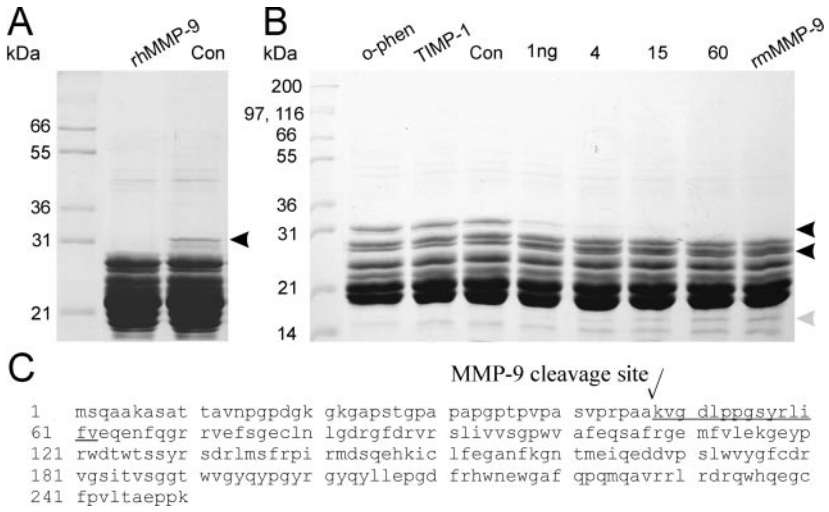

Figure 1. SDS-PAGE analysis of eye extracts incubated with MMP-9. *A)* A complete eye extract from a 9-wk-old mouse was incubated with rhMMP-9 or with carrier only (Con). Arrowhead indicates the 31 kDa protein degraded by MMP-9. Left lane shows the relative migration of molecular marker proteins, indicated in kilodaltons (kDa). *B)* Eye extract from a 2-wk-old mouse incubated with a dilution series of active rhMMP-9 (60ng, 15 ng, 4 ng, 1 ng), with carrier only (Con), with active rhMMP-9 plus TIMP-1, *o*-phenantroline (o-phen), or recombinant mouse MMP-9 (rmMMP-9). Black arrowheads indicate the intact 31 kDa substrate and the 24 kDa degradation product. The gray arrowhead indicates a γC crystallin degradation product. *C)* Amino acid sequence of mouse βB1 crystallin in one-letter code with an indication of the unique MMP-9 cleavage site. Underlined amino acids were identified by Edman degradation analysis.

experiments in which the MMP inhibitors *o*-phenantroline (5 mM) or TIMP-1 were added to the reaction mixture corroborated that this prominent cleavage was mediated by MMP-9. As expected, truncation of βB1 crystallin was observed after incubation with 10 ng active rmMMP-9. Using gels with a broad separation range, we detected the appearance of a 17 kDa band, which was identified by in-gel tryptic digest as a degradation product of γC crystallin.

Specific NH_2-terminal cleavage of βB1 crystallin

With the use of 2D-GE, an efficient separation of the lens proteome was obtained for intact and gelatinase B-treated samples. The most obvious change was the disappearance of the 31 kDa protein in lens homogenates and the appearance of a 24 kDa reaction product in gelatinase B-treated material. The NH_2 terminus of the 24 kDa protein was KVGDLPPGSYRLIFV, i.e., residues 48-62 of βB1 crystallin, which demonstrates that MMP-9 cleaves βB1 crystallin between residues Ala^{47} and Lys^{48} (Fig. 1*C*).

MMP-9 penetrates through the lens capsule and causes cataract

Since βB1 crystallin was identified as the major substrate (Fig. 1) and gelatinase B is reported to be a disease-associated enzyme (9, 10, 11), it was crucial to investigate whether pure gelatinase B can penetrate into the lens and cause opacification. We cultured lenses from 2-wk-old mice in minimal essential cell culture medium in the presence of 3 μg active rhMMP-9 for 16 h at 37°C. Within 15 min, an obvious opacity developed (**Fig. 2**). Incubation with MMP-9-free carrier did not alter the lens morphology. We systematically observed enzymatic loosening of the lens capsule in the presence of gelatinase B. Proteomic 2D-GE analysis of protein fractions of the incubated lenses demonstrated the disappearance of the 31 kDa substrate and the appearance of the 24 kDa reaction product as a prominent difference (Fig. 2). Again, the reaction product was identified by NH_2-terminal sequence analysis as the truncated βB1 crystallin. In these ex vivo incubations, the 17 kDa γC reaction product,

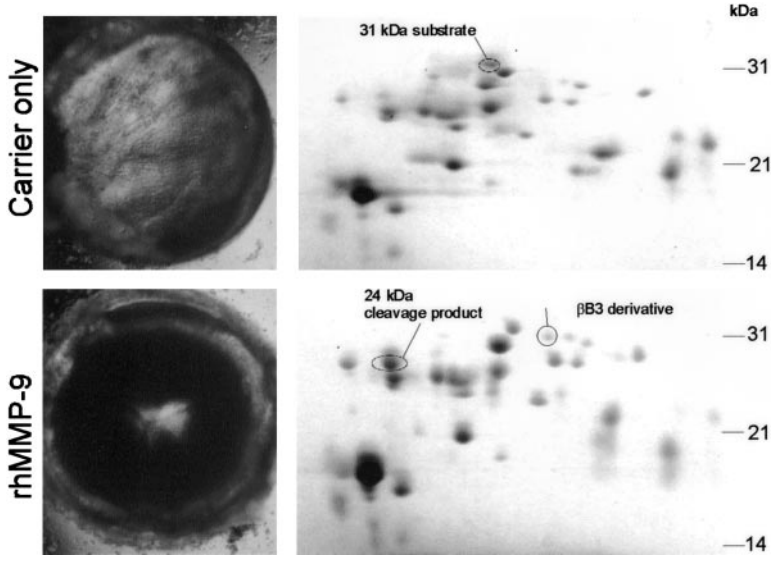

Figure 2. Ex vivo incubation of mouse lenses with active rhMMP-9. Lenses were photographed and subjected to 2D-GE analyses. The intact 31 kDa βB1 crystallin in the lens incubated with carrier only, the βB1 crystallin 24 kDa cleavage product, and a βB3 crystallin derivative in the MMP-9-treated lens are indicated.

observed in incubations of homogenates in vitro, was not formed. However, an additional minor crystallin modification (i.e., the appearance of a 29 kDa reaction product) was detected. This protein spot was identified as a derivative of βB3 crystallin.

βB1 Crystallin truncation occurs in vivo by endogenous gelatinase B activity

During cataract formation ex vivo, the appearance of the 24 kDa crystallin fragment was the major biochemical change detectable by proteomic analysis. Therefore, we attempted to visualize the 24 kDa crystallin fragment in vivo after intraocular injection of rhMMP-9. After injection of 1 μg gelatinase B into the vitreous chamber, the enzyme persisted for at least 1 h and the 24 kDa crystallin fragment was identified in lens homogenates (data not shown). The most crucial question—whether endogenous gelatinase B activity may result in cataract as a consequence of crystallin degradation—was addressed by local induction of active mouse enzyme. We used mouse CXC chemokine GCP-2, which is known to trigger progelatinase B secretion but has no apparent effect on constitutive gelatinase A (MMP-2) and TIMP secretion into the extracellular compartment. We injected mouse GCP-2 in nanogram quantities into the vitreous chamber of 2-wk-old mice to chemoattract granulocytes (12), which carry MMP-9 in specific granules. The chemokine induced a dose-dependent increase of endogenous progelatinase B in the injected eyes whereas gelatinase A (MMP-2) levels remained unaltered. Moreover, at a dosage of 500 ng GCP-2, activation of gelatinase B was detected by zymography analysis (**Fig. 3*A***). The 2D-GE analysis of lens extracts clearly and reproducibly showed a diminution of the intact 31 kDa βB1 crystallin and the appearance of the 24 kDa cleavage product, as confirmed by MALDI-TOF-TOF MS analysis (Fig. 3*B* and data not shown). To further prove that the cleavage is specifically dependent on gelatinase B, *MMP9*$^{-/-}$ mice were compared with *MMP9*$^{+/+}$ animals. When GCP-2 was injected in *MMP9*$^{-/-}$ mice, no alteration in MMP-2 levels was induced and, of course, gelatinase B was absent (Fig. 3). Moreover, degradation of the intact βB1 crystallin and the 24 kDa gelatinase B reaction product was not detected in any of the lenses of *MMP9*$^{-/-}$ mice (n=3) (Fig. 3).

Gelatin zymography analysis of tissue extracts is an ultrasensitive marker for neutrophil recruitment in wild-type mice, because neutrophils contain considerable amounts of uniquely gelatinase B. However, this technique is not applicable as a marker for neutrophils in MMP-9$^{-/-}$ animals. Hence, we are not sure whether neutrophil recruitment is impaired or normal in the MMP-9$^{-/-}$ animals in our model system. If it is impaired, we should not exclude the possibility that other molecules from neutrophils were responsible for βB1 crystallin degradation rather than neutrophil gelatinase specifically. To distinguish between these two possibilities, we incubated lens proteins with the intact cytosolic fraction of neutrophils or with cytosols depleted of MMP-9. A neutrophil cytosolic fraction that contained ~1 ng of gelatinase B (as determined by semiquantitative zymography analysis) specifically degraded βB1 crystallin, whereas the same amount of MMP-9-depleted neutrophil cytosolic fraction did not (data not shown). Consequently, we can conclude that gelatinase B was exclusively responsible for βB1 crystallin degradation in the in vivo model system.

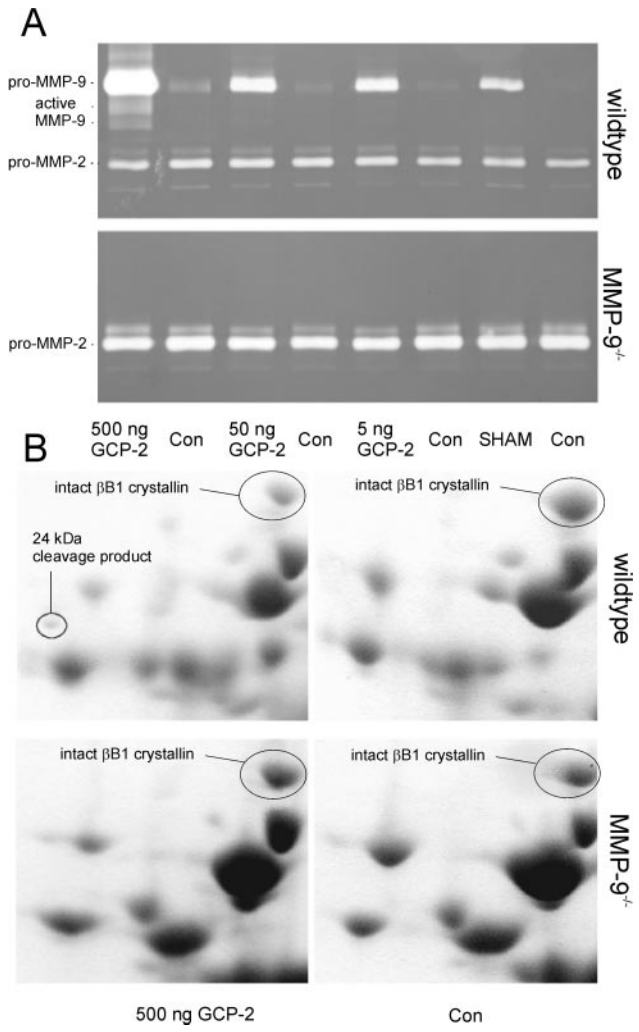

Figure 3. Intraocular changes after local up-regulation of endogenous MMP-9. *A)* Zymography analysis of eye extracts from wild-type *MMP9*$^{+/+}$ and *MMP9*$^{-/-}$ mice injected with various concentrations of GCP-2. A sham-injected sample was included; for each animal we analyzed the contralateral eye (Con). In the *MMP-9*$^{-/-}$ mice, only the constitutive MMP-2 is visible and the levels of this enzyme do not change under the pressure of GCP-2. In wild-type mice, however, gelatinase B is induced by GCP-2 in injected eyes in a dose-dependent way and is clearly activated in the eyes of mice injected with 500 ng GCP-2. *B)* 2D-GE analysis of lens extracts from wild-type *MMP9*$^{+/+}$ and knockout *MMP9*$^{-/-}$ mice injected with 500 ng GCP-2 and analysis of the contralateral eyes (Con). The 24 kDa βB1 crystallin cleavage product was observed only in lens extracts of eyes of wild-type mice after injection of GCP-2.

DISCUSSION

Proteolysis and subsequent precipitation of crystallin fragments are common underlying mechanisms of cataract. This has been demonstrated in rodent, human, and bovine lenses (13, 14). Originally, lens calpains were suggested to be responsible for proteolysis and subsequent insolubilization of lens proteins. Our results confirm lens proteolysis to be a cataract-causing factor, and we designate MMP-9 to be able to penetrate the lens and to cleave crystallins in vitro and in vivo. Nevertheless, fiber cells in the lens are tightly packed and the space between them is narrow and tortuous. Consequently, gelatinase B is not likely to diffuse from the outer surrounding ocular humors via a paracellular pathway. The lens capsule, the first barrier at the anterior side, should be conquerable since the main components, type IV collagen and laminin, are both supposed substrates for MMP-9 during an inflammation. Inside the lens, fiber cells communicate through gap junctions for the regulated passage of small molecules; for macromolecules, an alternative communication pathway has recently been described that is active in most of the lens tissue but not in the superficial layer of lens cells (15). Our data implicate that gelatinase B can cross this superficial cell layer. Since the mentioned communication pathways cannot account for this, it is most likely that the intrinsic activity of MMP-9 is essential here. In our in vivo model system, other leukocyte effector molecules may participate to overcome the mentioned barriers. Nevertheless, pure active MMP-9 was shown to be sufficient for penetration of the lens and cleavage of βB1 crystallin in vitro.

High levels of MMP-9 have indeed been found in the vitreous humor of inflamed human eyes (9, 10) and in mouse eyes with experimental endotoxin-induced uveitis (11). Ultraviolet (UV) light exposure, known to increase the risk of cataract (16, 17), is shown to induce gelatinase B release on fibroblast and corneal cells (18, 19) (**Fig. 4**). To date, a role for MMP-9 has been suggested in corneal repair and ulceration. Gelatinase B is up-regulated here upon engagement of the promoter by Pax6, a transcription factor that is up-regulated on its turn at the resurfacing corneal epithelium after corneal injury (20). Gelatinase B has been shown to play a role in retinal ganglion cell death after optic nerve ligation and in choroidal neovascularization (21). With regard to cataract formation, a role for gelatinases was suggested after the discovery of gelatinases in stressed lens epithelial cells (22).

As a model to recapitulate the role of increased MMP-9 in eye pathology, we induced endogenous mouse MMP-9 in vivo. We injected a chemokine to chemoattract MMP-9-bearing neutrophils. Among the CXC chemokines, IL-8/CXCL8 is the major neutrophil chemoattrant in humans (23). In mice, IL-8 does not exist, but GCP-2 compensates functionally. Like IL-8/CXCL8 in humans, mouse GCP-2 is a potent stimulus for degranulation and gelatinase B release (24, 12). Consequently, we used mouse GCP-2 to induce endogenous gelatinase B. Moreover, GCP-2 has been shown to stimulate the rapid release and activation of MMP-9 from granulocytes (12). The human counterpart IL-8 is shown to be induced by UV B light in cultured human corneal cells (Fig. 4) (25).

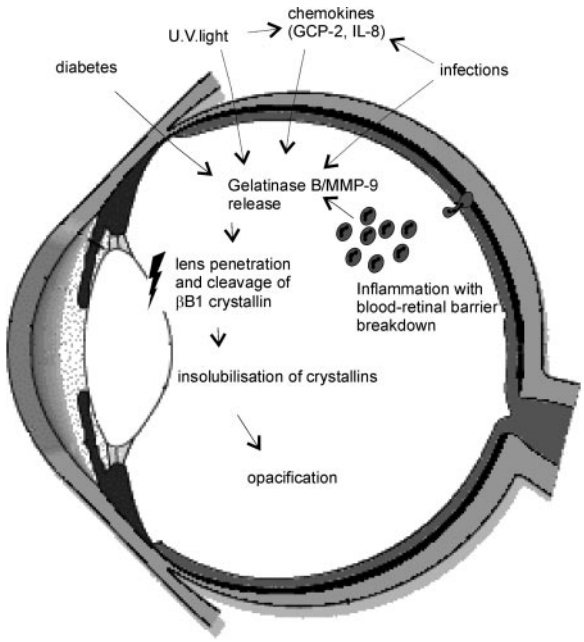

Figure 4. Gelatinase B/MMP-9 causes cataract in various diseases by cleaving lens crystallins. Increased levels of gelatinase B have been observed in various eye pathologies that lead to cataract. As a complication of diabetes, retinal disorder is a pathology in which increased levels of gelatinase B have been detected in the vitreous humor. Furthermore, acute and chronic infections lead to endogenous chemokine production, which attract inflammatory cells that break down the blood retinal barrier and enhance inflammatory reactions. Similarly, injection of the chemokine granulocyte chemotactic protein-2 (GCP-2) leads to local inflammatory cell recruitment and gelatinase B production. Finally, UV light has been shown to induce gelatinase B and IL-8, the human counterpart of GCP-2 in the mouse. Irrespective of the inducing agent, increased protease load in the eye, i.e., the balance between active MMP-9 and tissue inhibitors of matrix metalloproteinases, will cause penetration of gelatinase B into the lens and cleavage of βB1 crystallin and other crystallins. This causes local changes in the refractive index of the lens and opacification.

The cleavage of the βB1 crystallin protein substrate is the major biochemical change in lens extracts, as visualized by 2D-GE of ex vivo and in vivo samples. Yet we do not claim that the cleavage of βB1 crystallin is a unique action of MMP-9 in the pathophysiology of cataract. As discussed above, the cleavage of lens capsular proteins, like type IV collagen and laminin, may be important to allow access for the enzyme to the inner lens. The immunomodulating effect of gelatinase B through potentiation of chemokines like GCP-2 and cytokines like IL-1β or TNF-α may account for a positive feedback loop in terms of gelatinase B accumulation (26). On the other hand, degradation of βB1 crystallin may have further consequences in the eye. This crystallin indeed is present in nonpigmented epithelium of

the ciliary body and has been reported to be a major autoantigen in anterior uveitis (27). Since proteolysis of autoantigens with liberation of immunodominant epitopes has been described as a potential mechanism for autoimmunity induction (28), degradation of βB1 crystallin in the ciliary body could potentially lead to autoimmune uveitis.

Many phenotypes of MMP-9 deficiency are more pronounced in young mice than in adult animals (6, 29, 30). This may be caused by compensatory upregulation of other MMPs during life and, alternatively, by modification of gelatinase B substrates, as observed in this study with crystallins. Moreover, since βB1 crystallin degradation was more readily observed in lenses of young animals, our findings have a clinical correlate: children with uveitis have a high risk to develop cataract (31, 32, 33). Recently, a new case of experimental cataract was described in mice (34), where is was shown that mice deficient in the DNase II-like acid DNase gene are incapable of degrading DNA during lens cell differentiation, which results in the accumulation of undigested DNA in the fiber cells. In contrast to this rare genetically determined cause of cataract in mice, we designate more common diseases in humans as causes for cataract, including infections, UV light exposure during aging, and autoimmune diseases such as diabetes. As indicated in Fig. 4, in all of these human diseases increased gelatinase B levels may lead to cataract formation. In the short term, cataract may be formed acutely by severe infections, which cause neutrophil influx, eventually leading to hypopyon, and massive gelatinase B release. In the long term (e.g., during chronic inflammation, diabetes, or aging), small increases in gelatinase B levels compared with normal levels may gradually cleave lens crystallins and lead to slowly progressive cataract. In this way, a simple enzymatic reaction can be invoked for understanding cataract formation in a variety of diseases (Fig. 4). An understanding of the biochemical events and the availability of model systems are instrumental to define better ways of cataract prevention and therapy. FJ

This study was supported by the Fund for Scientific Research-Flanders (F.W.O.-Vlaanderen), the Concerted Research Actions of the Regional Government of Flanders (GOA11 2002-2006), the Cancer Research Foundation of Fortis AB, the Belgian Federation against Cancer, and the Charcot Foundation, Belgium. P.P. and P.V.D.S. are postdoctoral researchers of the Fund of Scientific Research-Flanders.

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Received for publication March 22, 2004.
Accepted for publication August 25, 2004.