Anatomy and Pathology/Oncology

Macrophage-Associated Gelatinase Degrades Basement Membrane at the Optic Fissure Margins During Normal Ocular Development in Mice

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PURPOSE. Basement membrane degradation and macrophage aggregation at the optic fissure margins are crucial to optic fissure closure during normal murine eye development. Basement membrane degradation is also an essential step in cancer development, and matrix metalloproteinases (MMPs) play an important role. In this study, we investigated MMP alteration at the degrading basement membrane of optic fissure margins in mice and attempted to clarify the relationship between MMP activity and macrophages.

METHODS. Serial coronal frozen sections of eyes from BALB/c fetuses were prepared and gelatinase activity was examined using in situ zymography techniques. The frozen sections were immunohistochemically stained with anti-F4/80, anti-MMP 2, and anti-MMP 9 antibodies. Serial coronal paraffin sections were also immunohistochemically stained with anti-type IV collagen and anti-F4/80, and basement membrane disintegration and macrophage aggregation at the optic fissure margins were examined.

RESULTS. The basement membrane of optic fissure margins was rapidly degraded during gestational days (GDs) 12.0 to 12.5. Meanwhile, gelatinase activity at F4/80-positive macrophages significantly increased during GDs 11.5 to 12.0 and declined thereafter; some of those were also positive for MMP2. The number of macrophages was also increased and decreased at nearly the same time.

CONCLUSIONS. Intramacrophage MMPs may be responsible for basement membrane degradation at the optic fissure margins during normal eye development in mice.

Keywords: basement membrane, optic fissure closure, macrophage, MMP, mouse

The secondary optic vesicle invaginates to become the optic cup and stalk. In normal mice, the optic fissure is usually closed late on gestational day (GD) 12.1–4 Previous reports have indicated that basement membrane degradation at the fissure margins is an essential step in the normal closure of the optic fissures in mice or hamsters.3–6 Moreover, many studies have reported evidence demonstrating that failure of basement membrane degradation prevents optic fissure closure in some strains of mice with ocular coloboma.7–9 Furthermore, partial or complete hypoplasia of ocular components (i.e., ocular coloboma) is regularly found in the confined area corresponding to the inferior one-half of the eyeball.4–8,10–14 Therefore, basement membrane degradation at the optic fissure margins is crucial to closure of the optic fissure during normal development; nevertheless, the mechanism(s) of action have yet to be determined.

Degradation of the basement membrane is also an essential step in invasive growth and metastasis in cancer, in which matrix metalloproteinases (MMPs) play an important role. These enzymes degrade the extracellular matrix, enabling cancer cells to migrate and proliferate. An in situ zymography technique using a fluorogenic dye-quenched (DQ)-gelatin substrate in unfixed cryostat sections has been introduced. The application of DQ-gelatin in combination with a gelled medium has enabled precise localization of gelatinolytic activity in cancer tissue.15–17 However, there have been few studies investigating MMP activity at or near the basement membrane during normal optic fissure closure.

Previous studies have demonstrated that macrophages are abundant around the optic fissure during normal optic fissure closure and that macrophage aggregation is disturbed in mice with ocular coloboma.7 Thus, it is possible that a reduced number of phagocytic cells can lead to a reduction in the concentration of MMP enzymes, which in turn may prevent basement membrane degradation in mice with ocular coloboma. However, there have been no studies investigating MMP enzymes at the fissure margins during normal optic fissure closure in mice.

The purpose of this study, therefore, was to confirm whether MMPs are expressed at or near degrading basement membranes during normal optic fissure closure in fetal murine eyes using an in situ zymography technique and to clarify the role of and relationship between phagocytic cells and MMPs in these tissues.

METHODS

Animals

All animal handling procedures and experimental protocols were in accordance with animal care guidelines established in...
Table 1. Summary of Experimental Design and Number of Eyes Examined

<table>
<thead>
<tr>
<th>Protocol</th>
<th>GD</th>
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<tr>
<td>Frozen sections, n</td>
<td>11.5</td>
</tr>
<tr>
<td>1. In situ zymography</td>
<td>8</td>
</tr>
<tr>
<td>2. In situ zymography + F4/80</td>
<td>8</td>
</tr>
<tr>
<td>3. F4/80 and MMP 2</td>
<td>8</td>
</tr>
<tr>
<td>4. F4/80 and MMP 9</td>
<td>8</td>
</tr>
<tr>
<td>Paraffin sections, n</td>
<td>12.0</td>
</tr>
<tr>
<td>5. Type-IV collagen</td>
<td>8</td>
</tr>
<tr>
<td>6. F4/80</td>
<td>8</td>
</tr>
<tr>
<td>7. Electron-microscope</td>
<td>8</td>
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<tr>
<td>specimens</td>
<td>8</td>
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the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Committee for Animal Experiments of Setsunan University (Osaka Prefecture, Neyagawa, Japan) and the Japanese Association for Laboratory Animal Science. BALB/cAnNCrlCrlj (BALB/c) mice were obtained from a commercial supplier (Charles River Japan, Inc., Kanagawa, Japan). This mouse line was maintained as an inbred strain by full sib-mating at the authors’ laboratory. For a prenatal study, 32 pregnant BALB/c mice were used to obtain these embryos. A total of 160 embryos were used for the present investigation. All animals were housed individually in polycarbonate cages in an air-conditioned animal room at a temperature of 20°C to 26°C, a relative humidity of 40% to 70%, a 12-hour/12-hour light/dark cycle, and ventilation with filtered fresh air, and they were allowed ad libitum access to water.

Preparation of Ocular Specimens

Female BALB/c mice older than 8 weeks of age were mated with males of the same strain (older than 12 weeks of age) from 1900 to 0800 hours (i.e., overnight). If a vaginal plug was detected the following morning, that day was designated day 0 of gestation. The pregnant females were euthanized on GD 11.5 to 13.0 to obtain developing embryos. Dams were deeply anesthetized and euthanized by exsanguination from the abdominal aorta; the uterus with embryos were immediately removed and subjected to investigation.

Experimental Design

The experimental design is summarized in Table 1. Serial coronal frozen sections of eyes from GD 11.5 to 13.0 fetuses were prepared at each stage, and gelatinase activity was examined using an in situ FITC-conjugated zymography technique (experimental design 1). Gelatinase activity was similarly observed for other serial coronal frozen sections of eyes from GD 12.0 fetuses; the same sections were used to observe macrophages using immunohistochemical staining with anti-F4/80 (experimental design 2). A separate set of serial coronal frozen sections of eyes from GD 12.0 fetuses were immunohistochemically stained with anti-F4/80 and anti-MMP 2 antibodies (experimental design 3), as well as anti-F4/80 and anti-MMP 9 antibodies (experimental design 4). Serial coronal paraffin sections of eyes from GD 11.5 to 13.0 fetuses were used for observations of basement membrane using immunohistochemical staining with anti-type IV collagen (experimental design 5), and F4/80-positive cells at the optic fissure margins were counted at the same stage using a separate set of serial coronal paraffin sections (experimental design 6). For electron microscopy, eyes from GD 12.0 fetuses were used (experimental design 7).

Table 2. Summary of Antibodies Used

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
<th>Antibody Clonality; Source</th>
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<tbody>
<tr>
<td>Anti-type IV collagen</td>
<td>1:2000</td>
<td>Rabbit polyclonal, LSL-LB-1403; Cosmo Bio Co., Ltd., Tokyo, Japan</td>
</tr>
<tr>
<td>Anti-F4/80</td>
<td>1:200</td>
<td>Rat monoclonal, MCA9497; AbD Serotec, Kidlington, United Kingdom</td>
</tr>
<tr>
<td>Anti-MMP 2</td>
<td>1:100</td>
<td>Rabbit polyclonal, ab37150; Abcam, Tokyo, Japan</td>
</tr>
<tr>
<td>Anti-MMP 9</td>
<td>1:150</td>
<td>Rabbit polyclonal, ab38898; Abcam, Cambridge, United Kingdom</td>
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Frozen Sections

Fetal heads were embedded in OCT compound (Sakura Finetek Japan, Tokyo, Japan) immediately after dissection, frozen in liquid nitrogen, and stored at −80°C until used. Serial sections (approximately 10 μm) were then cut at a cabinet temperature of −25°C using a cryostat (Leica CM3000; Leica Microsystems, Wetzlar, Germany) and dried in cold air for 5 minutes. The frozen sections were subjected to in situ zymography and immunohistochemical staining (Table 1).

Paraffin Sections

Immediately after dissection, the fetal mouse heads were fixed in 4% paraformaldehyde for 24 hours. The heads were then bisected and dehydrated in a sequential series of ethanol (50% to 100%) washes and embedded in paraffin. Coronal serial 4-μm sections were cut, and each was subjected to immunohistochemical staining (Table 1).

Electron Microscope Study of Basement Membranes at the Fissure Margins

For electron microscopy, three BALB/c embryos at GD 12.0 were used (Table 1). Embryonic heads were refixed using 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed in 1% osmium tetroxide solution (pH 7.4) for 2 hours, and processed into epoxy resin. Semi-thin sections were cut and stained with toluidine blue. Ultrathin sections were cut and stained with uranyl acetate and lead citrate, and then examined using an electron microscope (JEM1200EX; JEOL, Tokyo, Japan).

In Situ Zymography

Gelatinolytic activity was assessed in unfixed cryostat sections (approximately 10 μm) using DQ-gelatin as a substrate (Enz-Chek; Molecular Probes, Eugene, OR, USA) according to a modified in situ zymography method.18 Cryostat sections were air-dried for 10 minutes. DQ-gelatin was dissolved at a concentration of 1 mg/mL in water and then diluted 1:20 in 1% (w/v) low gelling temperature agarose (Nacalai Tesque, Inc., Tokyo, Japan) in PBS containing 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI) (1.0 μg/mL) to counterstain nuclei. The mixture (20 μL) was layered on top of the sections, which were subsequently covered with a coverslip. After gelling the agar at 4°C, incubation was continued for 24 hours at room temperature. FITC fluorescence in DQ-gelatin was detected using excitation wavelengths between 460 and 500 nm. DAPI was detected using...
Excitation wavelengths between 340 and 380 nm. Digital images corresponding to the area of the neural retina, including fusing optic fissure at the center (20x objective lens, 330 pixels [80 \mu m] × 412 pixels [100 \mu m], 8000 \mu m²), were captured using a digital camera (DC500; Leica Microsystems) attached to a light microscope (DM5500; Leica Microsystems). The sections were morphometrically analyzed by image processing and analysis software (IP Lab version 4.0; BD Biosciences, Rockville, MD, USA). The positive ratio of FITC fluorescence was calculated by dividing the FITC fluorescent area by the constant area of neural retina (8000 \mu m²). In each eye, the positive ratios of all slides were averaged, and the value was regarded as a representative datum.

Immunohistologic Analysis

The frozen sections were dried using cool air for 30 minutes and fixed using cold acetone for 10 minutes. The paraffin-embedded sections were deparaffinized in xylene and rehydrated through graded ethanol washes at room temperature; 0.05 M Tris-buffered saline (pH 7.6) with 0.03% Tween 20 (TBST) was used to prepare solutions, and sections were washed between various steps. Nonspecific endogenous peroxidase activity was blocked by exposure to 0.03% hydrogen peroxide in 100% methanol for 5 minutes, and masking was performed with 5% normal goat serum for 30 minutes at room temperature. Samples were incubated overnight at 4°C with primary antibodies (Table 2). The slides were subsequently rinsed with TBST, treated for 30 minutes at room temperature with simple mouse MAX PO (Rat or R) (Nichirei, Tokyo, Japan), rinsed with TBST, incubated in diaminobenzidine solution containing 0.01% hydrogen peroxide for the peroxidase coloring reaction, and counterstained with Mayer’s hematoxylin. Regarding the immunofluorescence double staining of MMP2/F4/80, incubation was carried out overnight at 4°C with an anti-MMP2 antibody and an anti-F4/80 antibody (diluted 1:100). The slides were subsequently rinsed with TBST, treated for 60 minutes at room temperature with Alexa Fluor 488- or 594–conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA), and rinsed with TBST. As a negative control, mouse or rat isotype immunoglobulin, diluted to the same concentration, was substituted for the primary antibody.19

Judgment of Optic Fissure Closure

The serial paraffin sections were prepared for immunohistologic analysis for type IV collagen. The basement disintegration ratio of optic fissure margins (i.e., the ratio of optic fissure closure) was calculated by dividing the length of the disappeared basement membrane by the retinal thickness at the fusing site. Thus, the ratio was considered 0% when the basement membranes of inner layer (presumptive neural retina) and outer layer (presumptive retinal pigmented epithelia) completely persisted (unfused). In contrast, the ratio was considered 100% when the two layers completely disappeared (fused), and there was no basement membrane at the fusing site (Fig. 1).

Macrophage Aggregation at the Optic Fissure Margins

The number of F4/80-positive cells at the optic fissure margins in the paraffin section was counted, and the mean was calculated.

Statistical Analysis

Wilcoxon rank-sum test was used to analyze the positive ratio of FITC fluorescence. A P value of less than 0.05 was considered to be statistically significant.
RESULTS

Morphologic Changes in the Basement Membrane

Both optic fissure margins closely approached one another but had yet to fuse (0%), and type IV collagen–positive basement membranes clearly persisted in all eyes at GD 11.5 (Fig. 1A). However, these basement membranes rapidly disintegrated, and approximately one-half of basement membranes lysed at GD 12.0 (Fig. 1B). Furthermore, basement membrane was scarcely observed at GD 12.5, and no basement membrane was observed at the fused ventral area of the optic cup in any eyes at GD 13.0 (Fig. 1C), resulting in normal optic fissure closure in BALB/c fetuses. As a result of morphometric examination, the ratio of basement membrane disintegration of optic fissure margins (optic fissure closure) was 0% at GD 11.5, and the value increased up to 43.9% at GD 12.0, 89.4% at GD 12.5, and 100% at GD 13.0 (Fig. 2).

An ultrastructural study revealed that the fissure margins approached one another on GD 12.0, and both basement membranes became apposed with multifocal contact. In areas where appositional contact developed, fragmented (white arrow) or disappeared (double arrow) basal lamina was alternately detected. Cytoplasmic process (*) is observed on cell lining the optic fissure at the foci of basement degradation. Basal lamina (black arrows).

Gelatinolytic Activity Detected Using DQ-Gelatin

The results of morphologic and morphometric study on FITC fluorescence at the area of optic fissure margins using in situ zymography with DQ-gelatin as substrate are shown in Figures 4 and 5. The mean positive ratio of FITC fluorescence was 2.54% and highest at GD 11.5, followed by that at GD 12.0 (1.22%); however, marked fluorescence (Figs. 4A, 4B) with higher positive ratios (>1%) was similarly recognized around the fusing optic fissure margins in six of eight eyes (75%) in

FIGURE 3. Electron micrograph of optic fissure margins in BALB/c fetus at GD 12.0. The fissure margins approach one another at GD 12.0, and both basement membranes become apposed with multifocal contact. In areas where appositional contact develops, fragmented (white arrow) or disappeared (double arrow) basal lamina is alternately detected. Cytoplasmic process (*) is observed on cell lining the optic fissure at the foci of basement degradation. Basal lamina (black arrows).

FIGURE 4. Change of gelatinolytic activity at the optic fissure margins in BALB/c fetuses. Marked fluorescence with higher positive ratios (>1%) was recognized around the fusing optic fissure margins at GD 11.5 (A) and at GD 12.0 (B); however, fluorescence became gradually less active at GD 12.5 (C) and GD 13.0 (D). Green, fluorescence reflecting positive gelatinase activity; blue, nuclei (DAPI); *, optic fissure, arrows, unfused optic fissure margins, arrowheads, fused site of optic fissure margins.
each group, and there was no significant difference between the two groups. Meanwhile, fluorescence became less active after that, and both of the mean positive ratios at GD 12.5 (0.53%) and GD 13.0 (0.33%) were significantly lower compared with those at GD 11.5 and 12.0 (Fig. 5). Thus, positive gelatinase activity mildly—but significantly—became altered with drastic basement membrane degradation at the optic fissure margins around the same time.

**Relationship Between Increased Gelatinase Expression and Macrophage Aggregation at the Optic Fissure Margins**

The area exhibiting strong gelatinase activity (Fig. 6A) was positive for F4/80 (Fig. 6B) in the same frozen sections, and the area was recognized as macrophage. The number of F4/80-positive cells increases and decreases around GD 12.5 near the optic fissure margins; however, these cells are scarcely evident after GD 13.0. Data are expressed as mean ± SD (C). Immunofluorescence double staining also reveals that intracellular granules within F4/80-positive cell (green) at the optic fissure margins are also positive (red) for MMP-2 (gelatinase A) (D).

However, to date, no study has demonstrated the involvement of MMPs in basement membrane degradation during normal optic fissure closure; consequently, possible relationships and mechanisms remain unknown. The present investigation demonstrated that gelatinase (i.e., MMP) activity was mildly but significantly higher at the optic fissure margins at GD 11.5 to 12.0 compared with those after GD 12.5 in BALB/c murine fetuses, which corresponded to rapid basement membrane degradation, with very little MMP activity after fusion of the optic fissure. This is strong evidence supporting the hypothesis that varying MMP activity is highly associated with basement membrane degradation at the optic fissure margins during normal development in mice.

Phagocytic cells reportedly aggregate at fusing optic fissure margins during normal eye development in mice. These findings are consistent with the changes in BALB/c embryos observed in this study. Furthermore, the timing of the increase in F4/80-positive macrophages corresponded with basement membrane degradation at the optic fissure margins. More importantly, according to the in situ zymography results, apparent MMP (gelatinase) activity was observed just on the cross section of F4/80-positive macrophages aggregating at the fusing optic fissure margins. Moreover, intracellular granules in some F4/80-positive
macrophages at the optic fissure margins were positive for MMP-2, which is believed to degrade type IV collagen, an important substrate component of basement membranes. Therefore, it is highly probable that macrophages carry MMP enzymes and aggregate at optic fissure margins to degrade basement membranes in normal murine embryos.

In this study, MMP activity was clearly evident near the basement membranes of the optic fissure margins, not at disintegrated basement membrane. Furthermore, MMP expression was limited to the inside of macrophages and was not detected outside macrophages. The concentration of active MMPs released from macrophages is expected to be lower than that of intracellular MMPs, and the effective concentration of MMPs in fetal ocular tissue may be significantly lower compared with MMPs in adult cancer tissue. Thus, it is possible that trace amounts of MMPs released from macrophages could not be detected using in situ zymography methods, even if MMPs are expressed solely in the basement membranes. Nevertheless, the precise mechanisms remain unknown.

In conclusion, increased MMP activity involving aggregated macrophages may be responsible for basement membrane degradation at the optic fissure margins during normal eye development in mice.

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References