Diminished corneal angiogenesis in gelatinase A-deficient mice

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Abstract The goal of the present study was to define the role of gelatinase A in angiogenesis. We performed corneal micropocket assays in gelatinase A-deficient mice and their age-matched wild-type littermates. The corneal neovascular area in gelatinase A-deficient mice (0.15 ± 0.14 mm^2) was significantly less than that of wild-type littermates (0.53 ± 0.35 mm^2; $P < 0.01$). Similarly, aortic ring assays showed significant reduction of endothelial outgrowth in gelatinase A-deficient mice (0.26 ± 0.14 mm^2) as compared to wild-type littermates (0.44 ± 0.06 mm^2; $P < 0.05$). These results suggest that gelatinase A may play an important role in the regulation of corneal angiogenesis. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Gelatinase A; Matrix metalloproteinase; Cornea; Angiogenesis; Knockout mouse

1. Introduction

Angiogenesis, the formation of new vessels from existing vessels, is a complex process, which involves the degradation of the vascular basement membrane and migration and proliferation of vascular endothelial cells. Extracellular matrix (ECM) proteolysis is now being appreciated as an important biochemical and cellular regulator of neovascularization, vascular morphogenesis and vascular invasion [1,2]. At the initial stages of angiogenesis, the pre-existing basement membrane is digested by endothelial enzymes such as matrix metalloproteinases (MMPs), allowing these cells to traverse the basement membrane. Thus, the regulation of angiogenesis may be dependent on the proper temporal and spatial expression of these enzymes within the vascular microenvironment.

MMPs are a family of zinc-containing, ECM-degrading enzymes that share common structural and functional properties. Since both gelatinase A (MMP-2) and gelatinase B (MMP-9) possess the ability to cleave type IV collagen, which is a major component of the basement membrane, these MMPs have been suggested to play a crucial role not only in wound healing [3,4,5] but also in angiogenesis [1,6,7].

In the present study, we aimed to define the physiological role of gelatinase A in the process of angiogenesis. We examined corneal angiogenesis induced by basic fibroblast growth factor (bFGF) in mice deficient in gelatinase A in vivo, to determine whether a null mutation in the gelatinase A gene leads to the suppression of angiogenetic response. Additionally, we prepared aortic rings from gelatinase A-deficient mice to determine the role of gelatinase A in vascular endothelial cell migration and tube formation in vitro.

2. Materials and methods

2.1. Animals

All animal studies were conducted in accordance with the Animal Care and Use Committee guidelines of the Massachusetts Eye and Ear Infirmary and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Gelatinase A-deficient mice and age- and strain-matched wild-type littermates 6–10 weeks of age were used. The generation of gelatinase A-deficient mice has recently been described in detail [6]. Heterozygous littermates were mated to obtain homozygous animals. Genotyping of animals was performed by polymerase chain reaction (PCR) of DNA obtained from tail biopsies. Primers for wild-type alleles were located in 5′ region (5′-TCC ACC CGG TGC TGC CAG CAC TCT TCC AGC CCA GC-3′) and exon 1 (5′-GCC GGG GAA CTT GAT GAT GG-3′), and primers for mutated alleles were located in PGK-neo cassette (5′-CTT GGG TGG AGA GGC TAT TC-3′ and 5′-AGG TGA GAT GAC AGG AGA TC-3′).

2.2. Corneal micropocket assay

A mouse corneal micropocket assay was carried out as previously described [8,9]. The mice were anesthetized by a combined ketamine and xylazine injection. Lidocaine eye drops were used for local anesthesia. Corneal micropockets were created with a modified von Graefe knife in gelatinase A-deficient mice ($n = 15$) and wild-type littermates ($n = 20$). Hydron pellets (0.4×0.4 mm) containing 50 ng of human bFGF (R&D Systems, Minneapolis, MN, USA) and 40 μg of sucrose aluminum sulfate were implanted into corneal pockets. Ofloxacin eye drops were instilled after surgery. The eyes were examined and photographed on the seventh postoperative day by slit lamp microscopy (Nikon, Tokyo, Japan). Color images were magnified 100× to allow precise measurement of corneal neovascularization. The neovascular area was calculated using Area (mm^2) = $0.2 \times \pi \times$ maximal vessel length (mm) × clock hours of neovascularization as described previously [10].

2.3. Light microscopic analysis

Mouse eyes, obtained on day 7 after bFGF pellet implantation, were fixed in 4% paraformaldehyde and embedded in paraffin. 3-μm-thick sections were cut and stained with hematoxylin and eosin. The sections were viewed and photographed with a Nikon Eclipse E800 microscope.

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Abbreviations: bFGF, basic fibroblast growth factor; ECM, extracellular matrix; FCS, fetal calf serum; MMP, matrix metalloproteinase; PCR, polymerase chain reaction

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2.4. Confocal laser scanning microscope

Wild-type mouse corneas, obtained on day 7 after bFGF pellet implantation, were frozen in OCT compound (Baxter Scientific, Columbia, MD, USA). Cryostat sections, 7 μm thick, were fixed in acetone for 10 min. After treatment with 1% bovine serum albumin, the sections were incubated for 1 h with rabbit anti-gelatinase A antibody (Oncogene, Manhasset, NY, USA), rat anti-CD31 antibody (Pharmingen, San Diego, CA, USA), and goat anti-type IV collagen antibody (Southern Biotechnology, Birmingham, AL, USA). Secondary antibodies used were fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (Jackson Immunoresearch Laboratories, West Grove, PA, USA), Cy5-conjugated anti-rat IgG antibody (Jackson Immunoresearch Laboratories) and rhodamine-conjugated anti-goat IgG antibody (Jackson Immunoresearch Laboratories). The sections were viewed with a Leica TCS 4D confocal laser scanning microscope (Leica, Heidelberg, Germany). Cy5 labelling originally generates a red fluorescent signal, which is depicted as blue for image analysis; this allows distinction between CD31 and the rhodamine-labelled collagen type IV.

2.5. Aortic ring assay

Aortic ring assays were performed as described by Nicosia et al. [11]. Briefly, aortas were obtained from gelatinase A-deficient mice or wild-type mice. Fatty tissues around the aorta were carefully removed under a surgical microscope. 1-mm-long aortic rings were cut and rinsed in five consecutive washes of Medium 199 (Life Technologies, Rockville, MD, USA). 48-well plates were coated with 150 μl of Matrigel (B.D., Bedford, MA, USA). After gelling at 37°C for 30 min, aortic ring was placed on its side on top of the gel and sealed in place with an overlay of 100 μl of Matrigel. After gelling at 37°C for 1 h, 300 μl of culture medium (Medium 199, Life Technologies) containing 5 ng/ml of bFGF (R&D Systems) with or without fetal calf serum (FCS) were added to each well. On day 7, the gels were photographed with a phase contrast microscope equipped with a digital Spot camera (Micro Video Instruments, Avon, MA, USA). The area of endothelial outgrowth was analyzed by manually encircling and computing square pixels by NIH image 1.62 software (NIH, Bethesda, MD, USA). The endothelial nature of the outgrowth was confirmed by immunostaining with CD31 antibody. Data are presented as mean ± S.E.M. Statistical significance was evaluated by using unpaired *t*-test. Differences were considered significant at $P < 0.05$.

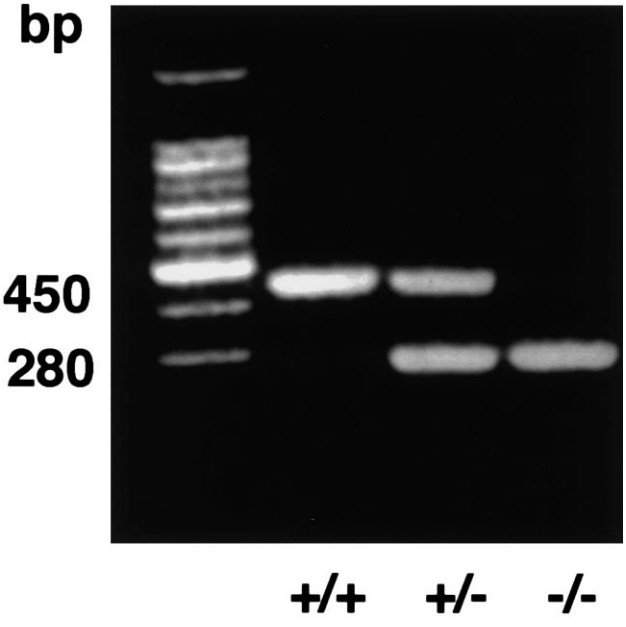

Fig. 1. PCR analysis of genomic DNA. The PCR fragments corresponding to normal alleles (450 bp) and targeted alleles (280 bp) are shown. +/+, wild-type; +/−, heterozygous; −/−, homozygous mutant.

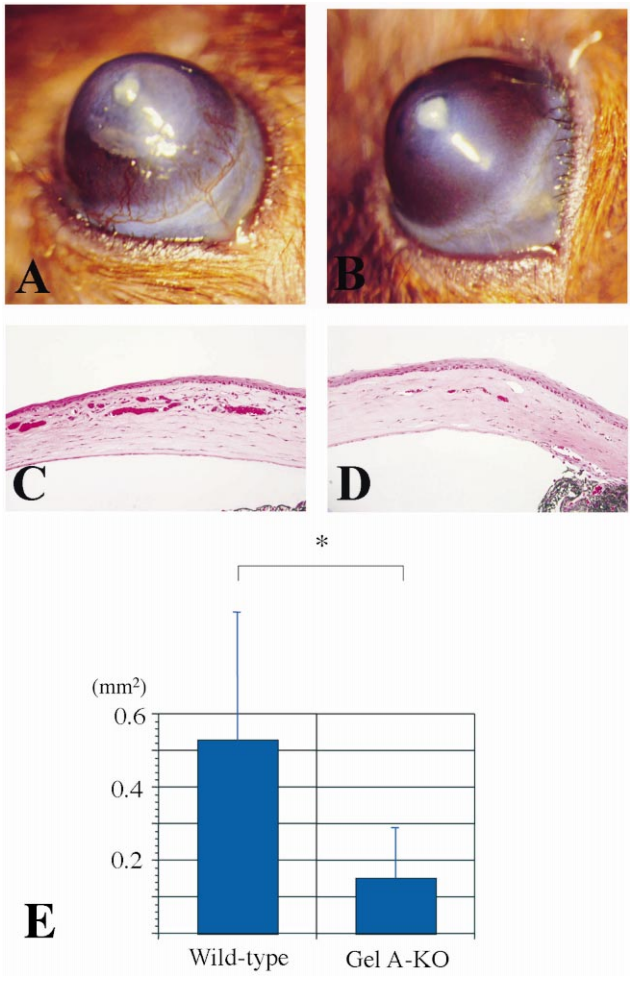

Fig. 2. Corneal neovascularization induced by bFGF. Pellets containing 50 ng of bFGF were implanted into corneas of wild-type littermates (A) and gelatinase A-deficient mice (B) and corneas were photographed with slit lamp microscope on day 7 after implantation. Hematoxylin and eosin staining of cornea from wild-type (C) and gelatinase A-deficient mouse (D) (magnification ×50). Corneal neovascularization was quantitated on day 7 after pellet implantation. Data represent mean values (± S.E.M.). *$P < 0.01$ (E).

3. Results

Homozygosity of gelatinase A-deficient mice used in our experiments was confirmed by genotyping of tail tip DNA using PCR (Fig. 1). Interbreeding of heterozygous mice gave rise to the expected Mendelian distribution of homozygous mutant mice (−/−), heterozygous (+/−) mice, and wild-type (+/+) mice.

3.1. Diminished bFGF response in corneas of gelatinase A-deficient mice

To determine whether corneal vascularization was altered in gelatinase A-null mice, micropellets of the slow release polymer-hydron containing bFGF were implanted into the cornea of gelatinase A-null mice and their wild-type littermates. The implanted corneas were photographed on day 7 after implantation (Fig. 2A and B). By day 7, 17/20 wild-type mice had visible corneal neovascularization extending more than 0.1 mm from the limbus, as compared to 4/15 in the gelatinase A-deficient group. The total area of neovascularization was

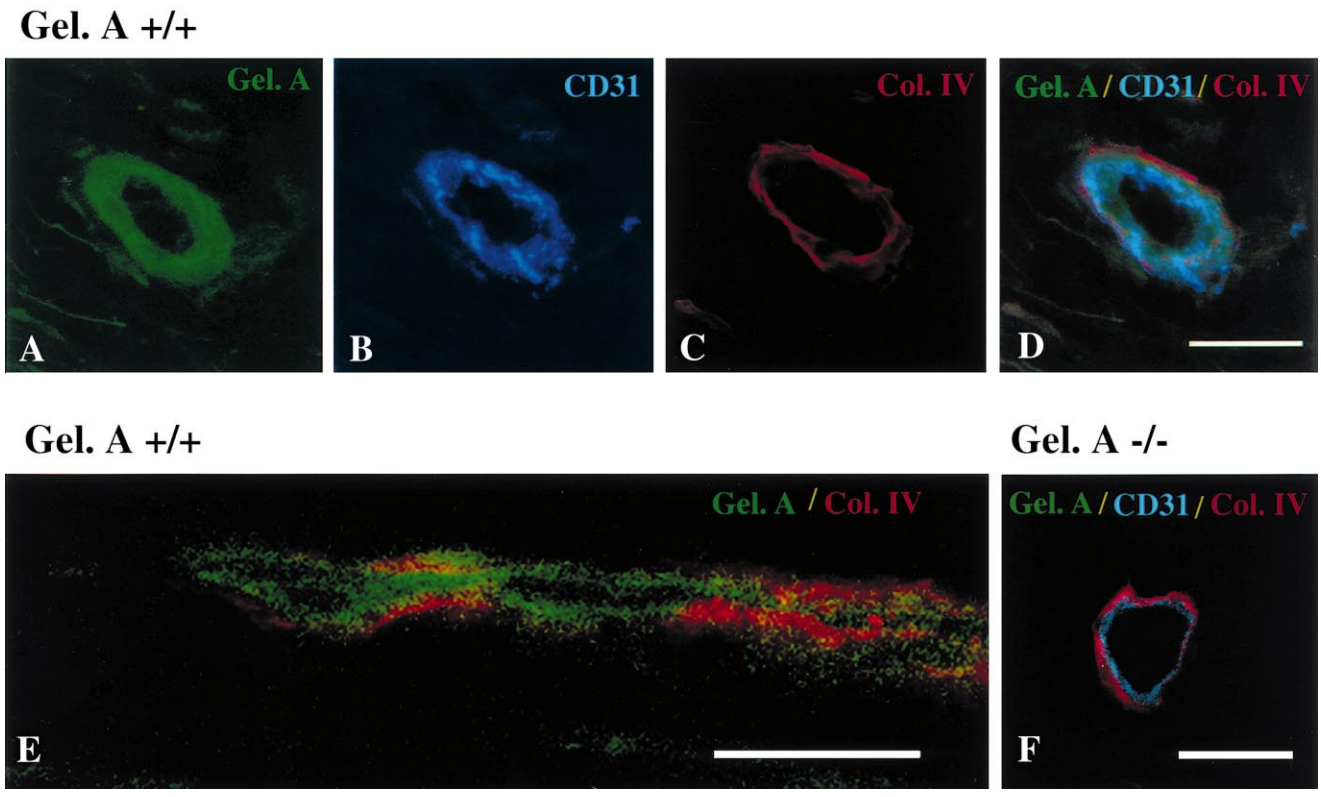

Fig. 3. Confocal micrographs of corneal neovascularization in wild-type mice (A–E) and gelatinase A-deficient mice (F). Immunohistochemical localization of gelatinase A (green) (A), CD31 (blue) (B), type IV collagen (red) (C), and triple staining (D) in wild-type mice. Triple staining confocal image demonstrates gelatinase A localization to the vascular endothelial region (D). Longitudinal section of corneal vessel confirming intraluminal localization of gelatinase A (green) as compared to surrounding localization of type IV collagen (red) in wild-type mice (E). Triple staining in gelatinase A-deficient mice (F). Bar: 10 μm.

calculated. The induced neovascular area was 0.53 ± 0.35 mm^2 in wild-type littermates and 0.15 ± 0.14 mm^2 in gelatinase A-deficient mice. The differences between these two groups were statistically significant ($P < 0.01$) (Fig. 2E).

Hematoxylin and eosin staining confirmed the presence of newly formed vessels in the mid-stroma in both wild-type and gelatinase A-deficient mice; the vessels were more prominent in the wild-type mice. No stromal edema and minimal inflammatory response were observed (Fig. 2C and D).

Triple staining confocal laser scanning microscopy in corneal new vessels enabled the simultaneous detection and localization of gelatinase A, CD31 and type IV collagen as shown in Fig. 3. The anti-CD31 antibody was used as a specific marker of the vascular endothelial cells. Expression of gelatinase A was represented by green (Fig. 3A), CD31 by blue (Fig. 3B), and type IV collagen by red (Fig. 3C). The confocal images suggested that gelatinase A was localized to the vascular endothelial region of corneal new vessels surrounded by type IV collagen in wild-type mice (Fig. 3D and E).

3.2. Reduced in vitro migratory activity of aortic endothelial cells of gelatinase-deficient mice

Decreased corneal neovascularization in gelatinase A-null mice and the positive staining of gelatinase A in wild-type vascular endothelial cells prompted us to explore whether the absence of gelatinase A affects endothelial cell migration in Matrigel. To examine this in vitro, an aortic ring assay was carried out. In the presence of 10% FCS, the difference between the area of endothelial sprouting in both groups was not significant (data not shown). However, endothelial outgrowth area in gelatinase A-null mice (0.26 ± 0.14 mm^2) was much less than that formed in wild-type littermates (0.44 ± 0.06 mm^2) after stimulation with 5 ng/ml bFGF (Fig. 4).

4. Discussion

Although it has become apparent that MMPs may play a role in the process of angiogenesis [1], little information is available concerning the in vivo mechanisms by which specific MMPs, such as gelatinase A (MMP-2), contribute to this process. In this report, we demonstrated that the angiogenetic response induced by bFGF is markedly reduced in mice lacking a functional gelatinase A gene compared with that of wild-type animals. Our data confirm the results reported by Elkin et al. [12] who showed that halofuginone, an inhibitor of collagen α1(I) and gelatinase A gene expression, almost completely suppressed bFGF-induced angiogenesis in vivo and inhibited endothelial capillary tube formation in vitro.

The use of MMP-deficient mice is potentially more advantageous because the distinct activities of the particular MMP would be eliminated. In addition, non-specific inhibition of ECM components and of other MMPs are minimized. Our data using gelatinase A-deficient mice provide more striking evidence for a critical role of this enzyme in angiogenesis.

The results of our study support the findings of Itoh et al. [6] studying tumor implantation in gelatinase A-deficient mice. They analyzed tumor growth and vascularization in the gelat-

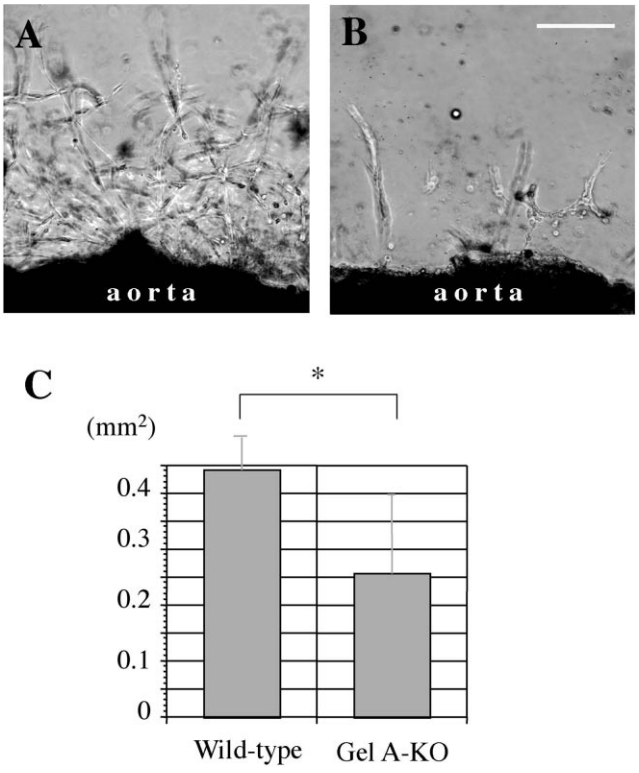

Fig. 4. Photographs of aorta ring assay. Aortic rings from wild-type littermate (A) and gelatinase A-deficient mouse (B) were cultured in endothelial growth medium containing 5 ng/ml bFGF without FCS. The difference in the endothelial outgrowth area was observed on day 7 post-explantation. Bar: 0.2 mm. Quantitation of microvessel outgrowth from mouse aortic rings (C). Graphs represent mean values (±S.E.M.) of eight samples in each group. $*P < 0.05$.

inase A-null host by using a dorsal air sac method and observed that tumor-induced angiogenesis in these animals was partially suppressed.

The precise mechanism by which gelatinase A influences angiogenesis is not clear. Our data from confocal microscopic examination strongly suggest that gelatinase A expression in vascular endothelial cells may play an important role during corneal neovascularization. To better understand how gelatinase A deficiency may lead to suppression of angiogenetic response, we conducted the aortic ring assays in Matrigel. A major component of Matrigel is type IV collagen, which is a substrate of gelatinase A. Our observations that endothelial cells from gelatinase A-deficient mice fail to display normal outgrowth in the presence of 5 ng/ml bFGF suggest that the differences in bFGF-induced angiogenesis between gelatinase A-deficient mice and wild-type may be related to differences in the vascular endothelial cells. It might be difficult for the endothelial cells lacking a functional gelatinase A to traverse the basement membrane. Therefore the degradation of basement membrane by gelatinase A may be an important event in the process of angiogenesis. However, we cannot exclude the possibility that gelatinase A may induce endothelial proliferation. This notion is consistent with previous studies showing suppressive effects of gelatinase A inhibitors (TIMP-2 and neutralizing antibodies to gelatinase A) on endothelial proliferation [13].

Zhou et al. [14] performed bFGF micropocket assays and showed complete absence of corneal angiogenesis in membrane type 1-MMP (MT1-MMP; MMP-14)-deficient mice. MT1-MMP contains a transmembrane domain, which facilitates the cell-mediated activation of gelatinase A. Thus, it is tempting to speculate that activation of gelatinase A by MT1-MMP is important for the regulation of angiogenesis [14].

Corneal micropocket assay in mice requires a relatively demanding technique, but offers a distinct advantage in investigating angiogenesis. Inflammation, as the major angiogenic stimulus, can be minimized using this model, and measurements of the neovascular response can be readily and noninvasively documented with slit lamp biomicroscopy [8].

The gelatinase A-null mice developed almost normally, and bFGF induced corneal angiogenesis even in the gelatinase A-mutant mice, clearly indicating that the angiogenetic process is not totally dependent on gelatinase A. One possibility that needs further investigation is that other MMPs such as gelatinase B (MMP-9) are upregulated in response to the absence of gelatinase A in the mutant mice. The production of gelatinase A −/−; gelatinase B −/− double-mutant mice may be valuable for testing this hypothesis.

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