Localization and expression of chondromodulin-I in the rat cornea

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Summary. The localization and expression in the rat cornea of chondromodulin-I (ChM-I), an inhibitory angiogenesis factor, were examined by immunohistochemistry, Western blot analysis, ribonuclease protection assay, and real-time PCR assay. We found immunoreactivity for ChM-I in the epithelial layer but not the stromal layer or endothelial layer in the cornea, in addition to the positive ChM-I immunoreactivity in other sites in the eye such as the sclera, retina, and ciliary body. The ChM-I immunoreactivity was most intense at the outside of the basal cells and in their cytoplasm while the intensity of the immunoreactivity decreased gradually from the wing cells to the superficial cells in the corneal epithelial layer. No reactivity however, was detected in the Bowman's membrane or conjunctival epithelial cells which had continuity with the corneal epithelial cells. The expression of ChM-I mRNA was demonstrated in the cornea at one-third less intensity than that in the sclera with choroids and retinal pigment epithelium by ribonuclease protection assay and real-time PCR. ChM-I in the corneal epithelial layer may prevent neovascularization and maintain avascularity in the cornea.

Introduction

Neovascularization in the eye is a leading cause of blindness. It usually occurs in association with severe ocular disorders, including diabetic retinopathy, prematurity retinopathy, choroidal neovascularization, and corneal neovascularization. Angiogenesis in the eye is believed to occur when angiogenesis factors such as basic fibroblast growth factor (Schweigerer *et al.*, 1987; Yamamoto *et al.*, 1996), vascular endothelial growth factor (Aiello *et al.*, 1994; Pierce *et al.*, 1995; Gerhardinger *et al.*, 1998) and transforming growth factor- β (Lutty *et al.*, 1991; Ogata *et al.*, 1997) overwhelm inhibitory angiogenesis factors such as angiostatin (O'Reilly *et al.*, 1994), endostatin (O'Reilly *et al.*, 1997), pigment epithelium derived factor (Ortego *et al.*, 1996; Dawson *et al.*, 1999), and tenomodulin (Oshima *et al.*, 2003).

Although no vasculature is physiologically present in corneal tissues, inflammatory, infectious, degenerative, and traumatic disorders in the cornea often induce neovascularization and lead to visual disturbance. These findings suggest that inhibitory angiogenesis factors serve to promote or suppress vascularization. However, the pathogenesis of corneal angiogenesis has not yet been clearly defined, and the identification and significance of actual angiogenesis or inhibitory angiogenesis factors remain unknown.

Chondromodulin-I (ChM-I) is a glycoprotein isolated from bovine epiphyseal cartilage as a growth stimulating molecule for chondrocytes (Hiraki *et al.*, 1991). This glycoprotein stimulates the proliferation, proteoglycan synthesis, and colony formation of growth plate chondrocytes in culture (Inoue *et al.*, 1997). Following its discovery, ChM-I was found to inhibit the DNA synthesis and tube formation

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of vascular endothelial cells (Hiraki *et al.*, 1997b), and tumor growth (Hayami *et al.*, 1999). The expression of ChM-I has been demonstrated in organs or tissues other than cartilage (Shukunami *et al.*, 1999; Funaki *et al.*, 2001). We found ChM-I expression in the eye and confirmed its localization at the ciliary body, ganglion cell layer of the retina, retinal pigment epithelium (RPE), and sclera (Funaki *et al.*, 2001). Because a structural analogy has been noted between the cartilage and cornea in terms of their avascularity, it may be speculated that some inhibitory angiogenesis factors may be present in the cornea to maintain the avascular condition.

In this study, we examined both the gene expression of ChM-I in the cornea by real-time PCR and ribonuclease protection assay as well as the localization of its protein by Western blot and immunohistochemical analysis.

Materials and methods

Animals and tissues

The animals used in our experiments were Brown Norway rats at 8 weeks of age. They were anesthetized with diethyl ether and sacrificed by cutting the axillary artery. For immunohistochemistry, the eyes were removed and fixed in methyl-Carnoy fixative for 16 h, dehydrated in graded ethanol, embedded in paraffin, and sectioned at 4 μ m on poly-L-lysine-coated slides.

For protein and RNA extraction, the eyes were dissected into several compartments under a microscope: the conjunctiva, cornea, iris/ciliary body, lens, retina without RPE, and sclera with choroids and RPE. As a positive control, articular cartilage was removed from newborn rat ribs. Total cellular RNA was purified by a modified acid guanidinium thiocyanate phenol-chloroform extraction method (TRIzol; Invitrogen Corp., CA, USA).

Immunohistochemistry

The paraffin-embedded eye sections were deparaffinized with xylene and rehydrated through graded ethanol and distilled water. They were incubated with 10% normal goat serum for 30 min, and incubated with a rabbit anti-rat ChM-I antibody diluted at 1:500 for 1 h at room temperature. The antibody was raised in rabbits by immunizing with a synthetic polypeptide (NH₂-PSTTRRPHEPRGNAGP-GRLSNRTRP-COOH) corresponding to amino acids 222-247 of rat ChM-I (Funaki *et al.*, 2001). After being rinsed in 0.1M PBS, the sections were reacted with a horseradish peroxidase-conjugated goat-anti-rabbit IgG antibody (1:5 dilution, Envision; DAKO, Kyoto) for 30 min at room

temperature, followed by treatment with True Blue (Kirkegaard & Perry Laboratories, USA). The sections were counterstained with Contrast Red (Kirkegaard & Perry Laboratories).

Western blot analysis

Reactivity of the ChM-I antibody and ChM-I contents in the eye compartments were examined by Western blot analysis. In brief, samples were extracted from the cornea, retina without RPE, sclera with choroids and RPE in a lysis buffer (8M urea, 0.8% Triton X-100, 0.2% sodium dodecyl sulfate, 3% 2-mercaptoethanol) and maintained at 4°C for 1 h. The homogenates were centrifuged at 15,000g for 15 min at 4°C, and protein concentrations of the supernatant were assayed by Lowry's methods (Peterson, 1997). For sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the supernatant samples were mixed with an equal volume of Laemmli sample buffer and boiled for 5 min. Proteins (10 μ g/lane) were run on 12.5% polyacrylamide slab gels and transferred to PVDF membranes (Amersham Pharmacia Biotech, Tokyo). The membranes were then blocked with 5% skimmed milk in PBS with 0.5% Tween 20 for 1 h at room temperature, and incubated overnight with the rabbit anti-rat ChM-I antibody (Funaki *et al.*, 2001) diluted at 1:2000 for 16 h at 4°C. After washing in PBS with 0.5% Tween 20, the membrane was incubated with the horseradish peroxidase-conjugated goat-anti-rabbit IgG antibody (1:1000, Envision; DAKO, Kyoto). The immunoreactivity was visualized using the ECL plus system (Amersham Pharmacia Biotech, Tokyo). As a positive control we used the protein (2.5 μ g) extracted from rat articular cartilage at 4 weeks of age (Kitahara *et al.*, 2003).

Ribonuclease protection assay

The expression of ChM-I mRNA was examined in the eye compartments by ribonuclease protection assay as reported previously (Sambrook *et al.*, 1989; Yamamoto *et al.*, 1994). Ribonuclease protection assay was conducted using ³²P-labeled antisense cRNA probes for ChM-I mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The templates were ChM-I cDNA (392 bp corresponding to nucleotides 868-1259 of the full-length cDNA) or GAPDH cDNA (114bp corresponding to nucleotide 674-787) as a housekeeping gene. An amount of 5 μ g of the total RNA isolated from the eye compartments and cartilage was hybridized with these probes, and unhybridized probes were digested with ribonucleases A and T1. Those probes protected from the ribonuclease digestion were electrophoresed on 6% polyacrylamide gels, and the dried gels were exposed to x-ray films (Fuji Film, Kanagawa) for 7

days at -80°C . Detection and analysis of the bands were performed by phosphor imaging techniques using Molecular Image FX (Bio-Rad Laboratories, Hercules, CA, USA). The results were represented as the ratio of integrated density for the ChM-I mRNA band to that for the GAPDH mRNA band.

Real-time PCR assay

Total RNA samples ($2\text{ }\mu\text{g}$ each) purified from sclera with choroids and RPE, retina without RPE, and cornea were reverse transcribed by using a kit (Superscript II Reverse Transcriptase cDNA Synthesis; Invitrogen Corp., CA USA). Real-time quantitative PCR was performed using a fluorescein PCR detection system (LightCycler, Roche Molecular Biochemicals, Indianapolis, IN, USA). The reactions were performed in a volume of $20\text{ }\mu\text{l}$ containing 10 pmol each of oligonucleotide primers, $2\text{ }\mu\text{l}$ of green fluorescent dye (LightCycler DNA Master SYBR Green I; Roche Molecular Biochemicals), Taq DNA polymerase, and dNTP mixture in reaction buffer. The primer sequences for real-time PCR assay of rat ChM-I mRNA were as follows: the forward primer, 5-AAGCAGTGCTCCCTCTACCA-3, and the reverse primer, 5-AAGGCCACGGGTAGTAACC T-3. For PCR assay, initial denaturation was done at 95°C for 5min, followed by 40 cycles of 95°C for 15 sec, 55°C for 5 sec, 72°C for 10 sec, and then slowly heated again from 65°C to 95°C to obtain the melting profile of the preparation. The house keeping gene, GAPDH served as an internal control for the quality of extracted RNA. The forward and reverse primer sequences of rat GAPDH mRNA were 5-CAAGATGGTGAAGGTCGGTG-3 and 5-CAAGATGGTGAAGGTCGGTG-3, respectively. The cycle number at which the signal was detected was used to determine the concentration of the cDNA from the GAPDH standard curve.

Results

Immunohistochemistry of ChM-I in the cornea

The localization of ChM-I protein in the rat cornea was studied by immunohistochemistry. Immunoreactivity for ChM-I was found in the corneal epithelial layer but not in the stromal and endothelial layers (Fig.1A). The intensity of the immunoreactive ChM-I decreased gradually toward the cornea-conjunctiva junction (corneal limb) (Fig.1B), and no immunoreactivity was detected in the conjunctival tissues (Fig.1C). In the corneal epithelial layer, the immunoreactivity was intense around and in the basal cells, but was less intense in the wing cells and superficial cells (Fig. 2).

Intense reactivity was demonstrated between basal cells locating on Bowman's membrane and in a part of their cytoplasm. No immunoreactivity for ChM-I was detected in keratocytes in the stromal layer, endothelial cells, Bowman's membrane, or Descemet's membrane of the cornea.

Western blot analysis

The presence of ChM-I protein in the cornea was examined by Western blot analysis. A single band of $\sim 32\text{ kDa}$ was immunoblotted in the corneal sample (Fig.3). Bands of similar size were also detected in the sclera without choroids and RPE, and retina with RPE, as reported previously (Funaki *et al.*, 2001), indicating that mature form of ChM-I was present in the cornea as shown in the sclera and retina. A group of multiple bands of $\sim 25\text{ kDa}$ immunodetected in the articular cartilage were presumed to correspond to various glycosylated forms of ChM-I (Kitahara *et al.*, 2003).

Ribonuclease protection assay

The expression of ChM-I mRNA was examined in the eye compartments by ribonuclease protection assay (Fig.4). ChM-I mRNA was detected in the cornea as previously reported in the sclera with choroids and RPE, and iris/ciliary body. No expression was detected in RNA samples from the lens, conjunctiva, and retina without RPE. The ratio of ChM-I to GAPDH mRNA expression was quantified by phosphor imaging techniques (Table 1). The ratios of ChM-I to GAPDH mRNA were 1.68×10^{-3} in the cornea and 5.77×10^{-3} in the sclera. ChM-I mRNA was expressed in the cornea, but this level was lower than in the sclera with RPE.

Real-time PCR assay

Quantitative analysis of ChM-I mRNA expression in the cornea was performed by real-time PCR assay (Table 1). Typical melting curves for GAPDH mRNA and ChM-I mRNA are shown in Figure 5. No secondary peaks were observed, indicating that target genes were specifically amplified by the assay. In the cornea, the ChM-I/GAPDH ratio was $8.02 \times 10^{-3} \pm 0.20 \times 10^{-3}$. In the sclera with choroids and RPE, the ChM-I/GAPDH mRNA ratio was $23.41 \times 10^{-3} \pm 3.35 \times 10^{-3}$ (mean $\pm$ SD) and $1.38 \times 10^{-3} \pm 0.09 \times 10^{-3}$ in the retina without RPE. The expression of ChM-I mRNA in the cornea was roughly one-third less than that in the sclera with choroids and RPE.

Fig. 1. Immunohistochemical localization of ChM-I in the rat cornea. **A:** The localization of ChM-I is restricted in the epithelial layer (Ep) but not in the stroma (S) or endothelial layers (En) of the cornea. **B:** Immunoreactivity for ChM-I detected in the corneal epithelial layer is reduced toward the corneal limb (CL) (cornea-conjunctiva junction). **C:** No ChM-I immunoreactivity is demonstrated in the conjunctival tissues. Ep: conjunctival epithelium. $\times 160$

Fig. 2. Cellular localization of ChM-I in the epithelial layer. At high magnification, immunoreactive ChM-I appears localized around the basal cells (B) and also in their cytoplasm. The immunostaining is weaker in the wing cells (W) and the superficial cells (S) in the corneal epithelial layer. K: keratocyte, E: endothelial cell, BM: Bowman's membrane, St: stroma, DM: Descemet's membrane. $\times 760$

Fig. 3. Western blot analysis of ChM-I. A broad band corresponding to a glycosylated form of ~25 kDa is detected in the articular cartilage sample (lane 1). A single band of ~32 kDa is detected in the samples from the cornea (lane 2) as well as the sclera with choroids and retinal pigment epithelium (RPE) (lane 3), and retina without RPE (lane 4).

Fig. 4. Ribonuclease protection assay for ChM-I mRNA in the eye compartments. ChM-I mRNA expressed in the articular cartilage as a positive control (lane 1) is significantly detected in the cornea (lane 7) as well as the iris/ciliary body (lane 4), sclera with choroids, and retinal pigment epithelium (RPE) (lane 5). No expression of ChM-I mRNA is demonstrated in the conjunctiva (lane 2), lens (lane 3), or retina without RPE (lane 6) and tRNA sample as a negative control (lane 8).

Discussion

This study was the first to detect ChM-I, an angiogenesis inhibitory factor, in the rat cornea at mRNA and protein levels and clarify the localization in the epithelial layer of the cornea. The presence of ChM-I and its physiological roles in angiogenesis inhibition have been demonstrated in the eye (Funaki *et al.*, 2001). The inhibition of retinal endothelial cell tube formation was also demonstrated by treatment with recombinant ChM-I in culture (Funaki *et al.*, 2001). In addition, a decrease in the capillary proliferation rate in the cornea was shown by the implantation of cartilage (Brem and Folkman, 1975). Thus, we consider that

ChM-I may play a role in the maintenance of avascularity in the cornea.

In the cornea, both angiogenesis factors and their inhibitors have been demonstrated. Angiostatin, endostatin, tenomodulin, and the pigment epithelium derived factor have been detected in the cornea as inhibitory angiogenesis factors, and the fibroblast growth factor and vascular endothelial growth factor as angiogenesis factors. The present study proposes that ChM-I is another inhibitory angiogenesis factor in the cornea, and that its avascularity is maintained by a balance between the angiogenesis factors and their inhibitors. Corneal neovascularization signifies the growth and extension of vessels toward the cornea from the corneal limb. The limbal vessels are normally prevented

Table 1. Quantitative analysis of expression levels of ChM-I mRNA in the eye compartments.

	Ribonuclease protein assay	Real time PCR assay
Sclera	5.77×10^{-3}	$23.14 \times 10^{-3} \pm 3.35 \times 10^{-3}$
Cornea	1.68×10^{-3}	$8.02 \times 10^{-3} \pm 0.20 \times 10^{-3}$
Retina	undetectable	$1.38 \times 10^{-3} \pm 0.09 \times 10^{-3}$
Ciliary body	0.27×10^{-3}	ND
Conjunctiva	undetectable	ND
Lens	undetectable	ND

The results are represented as the ChM-I/GAPDH mRNA ratios. Data are expressed as mean or mean $\pm$ SD. ND: not done.

Fig. 5. A representation of melting curves shown by a LightCycler. Typical melting curves are obtained for GAPDH and ChM-I cDNA synthesis. The samples used are the sclera with choroids and retinal pigment epithelium (RPE), cornea, and retina without RPE. Red line: GAPDH. Blue line: ChM-I

from entering into the cornea probably by growth-inhibiting substances in the cornea (Ashton *et al.*, 1953). Therefore, neovascularization could arise through a reduction in the inhibitory angiogenesis factors or the enhanced production of angiogenesis factors at the site. Although the entrance of new vessels into the cornea may happen at several corneal layers, the chief portal is at the upper and mid-third areas of the anterior stroma (57%), or between the epithelium and Bowman's layer (8.7%) (Cursiefen *et al.*, 1998). In the present study, ChM-I immunoreactivity was only localized in the corneal epithelial cells, indicating that at least ChM-I is present at the forefront where the cornea likely faces the danger of vascular invasion. In inflammatory, infectious, degenerative, or traumatic disorders of the cornea, ChM-I may be reduced at expression and protein levels, resulting in the onset of corneal vascular invasion. On the other hand, neovascularization may require not only the down-regulation of inhibitory angiogenesis factors, but also the up-regulation of angiogenesis factors (Folkman and Shing, 1992). It was recently shown that the vascular endothelial growth factor was up-regulated in inflamed and vascularized corneas in humans and animal models (Philipp *et al.*, 2000; Cursiefen *et al.*, 2000). Further studies are therefore needed to clarify the relationship between ChM-I and angiogenesis factors or other inhibitory angiogenesis factors in the cornea.

ChM-I has been presumed to be synthesized as a precursor form of ~37 kDa and secreted as a glycosylated mature form of ~25 kDa in the articular cartilage (Hiraki *et al.*, 1991). Western blot analysis in the present study immunoblotted a ~32 kDa band in the cornea and also in the retina and sclera. The molecular mass was smaller than the precursor form and larger than the glycosylated mature form isolated from the articular cartilage, suggesting that the ~32 kDa ChM-I might be a form which has been liberated from the precursor (~37 kDa) by truncation at a site different from that for the production of the cartilage mature form (~25 kDa) or a form differently glycosylated. Although we previously detected a major band of ~37 kDa and a minor one of ~30 kDa ChM-I in the rat retina (Funaki *et al.*, 2001), the ~37 kDa form was not detected in this study but a ~32 kDa form of ChM-I was demonstrated instead. The ~32 kDa form may be identical to the ~30 kDa one, but reason why we could not detect the ~37 kDa band is unknown.

In conclusion, we demonstrated that the cornea is also a site for the synthesis of ocular ChM-I. It is interesting that ChM-I is expressed in avascular sites of the eyes, such as the cornea. Because ChM-I has been shown to have an antiangiogenic activity, the presence of ChM-I in the cornea leads us to speculate that ChM-I functions as an antiangiogenic molecule there. ChM-I may prevent the

extension of vessels from the corneal limb, because ChM-I is located only in corneal epithelial cells facing the limbal vessels there. The expression of ChM-I in corneal disease-associated neovascularization should be investigated to clarify the precise roles of this molecule in the cornea.

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