

Morphological and Biochemical Analysis of Intact and Opaque Cornea in Dogs

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Summary: The arrangement of collagen fibrils and glycosaminoglycans (GAGs) in substantia propria are important for maintaining transparency of the cornea. Interferences in collagen fibrils and GAG production could be adversative to corneal integrity. In this study, six dogs consisting of four Beagles with normal cornea (normal), one Beagles with opaque cornea (sample No.1) and one Shih Tzu with neovascularization opaque cornea (sample No.2) were used. All samples were observed morphologically by light and electron microscopes to obtain diameter and distribution of collagen fibrils in substantia propria and were performed biochemically to investigate into GAGs and collagen types. The average diameter of collagen fibrils in the intact cornea of normal, sample No.1 and No.2 was 33.2, 35.0 and 25.0 nm, respectively. The percentage of matrix per unit area was 67% in normal, 87% in sample No.1 and 28.3% in sample No.2. The type III collagen ratio was 25.3% in normal, 21.3% in sample No.1 and 35.8% in sample No.2. The relative amount of heparan sulfate, chondroitin sulfate, dermatan sulfate and keratan sulfate was 1.5, 9.7, 51.1 and 37.7% in normal, 3.3, 26.0, 45.7 and 23.7% in sample No.1 and 1.2, 18.0, 16.6 and 54.1% in sample No.2. Hyaluronic acid was found only in sample No.1 with a relative amount of 1.3%. Since there was some relationship between collagen formation and GAGs composition, it might be speculated that disturbance in arrangement of collagen fibrils and GAG metabolism especially in substantia propria would bring up opacity of the cornea.

Introduction

Cornea is mainly composed of anterior epithelium, substantia propria and posterior endothelium. In these main three layers, especially in the substantia propria, collagen types, amount and arrangement of cells, formation of collagen fibrils and components type of extracellular matrices are indispensable for transparency of cornea^{1,2,4}. Major extracellular matrix components are various types of glycosaminoglycans (GAGs) linked to their core proteins with proteoglycans. GAGs consist chiefly of chondroitin sulfate, dermatan sulphate, heparan sulfate and keratan sulfate². Among these, while keratan sulfate plays a principal role in providing corneal clearness and maintaining a constant diameter of collagen fibril and interfibrillar space^{2,6,7}, dermatan sulphate inhibits the in-

crease of collagen fibril diameter^{8,11}. Metabolic disorder of GAGs was reported to bring out collagen fibril malformation¹². As mentioned above, cloudiness of the cornea could be related to the abnormality of both cellular and non-cellular components, especially in the substantia propria. However, little attention to this particular aspect has been given to the point. In this study, clarification of the corneal opacity is to analyze morphology of collagen fibrils in the substantia propria together with biochemistry of some major extracellular substances.

Materials and Methods

All experiments followed the protocols approved by the Ethics Committee of Rakuno Gakuen University,

Japan, and compliance with the NIH Guide for the Care and Use of Laboratory Animals [DHEW (DHHS) Publication No. (NIH) 85-23, revised 1985, Office of Science and Health Reports, DRR/NIH, Bethesda, MD 20205].

Animals and sample sources

Five Beagles (four with normal cornea and one opaque cornea) purchased from Kitayama Labes, Nagano, Japan, were used in this study. The Beagle dogs were euthanized by a verdose anaesthetic injection of with thiopental. Cornea was surgically removed immediately postmortem. Due to corneal angiogenesis, the Shih Tzu dog had undergone keratoplastic surgery in Kushiro Animal Hospital, and then angiogenesis cornea was removed after the surgical operation. The opaque cornea from the Beagle dogs was assigned as sample No.1 and the opaque cornea with regenerating blood vessels of the Shih Tzu dog was assigned as sample No.2.

Electron microscopy

The corneas were carefully sliced into blocks of $1 \times 1 \times 5$ mm under a dissecting microscope and immersively fixed in 3.0% glutaraldehyde in 0.1 M phosphate buffer. For transmission electron microscopy (TEM), the glutaraldehyde-fixed samples were further treated with 1.0% osmium tetroxide for 1 hr at room temperature. The samples were dehydrated in a graded ethanol series, embedded in Queto1 812 resin (Nissin EM, Tokyo, Japan), sectioned with a diamond knife, stained with 1.0% uranyl acetate and 1.0% lead citrate and examined under a TEM (JEM-1220; JEOL, Tokyo, Japan) at an acceleration voltage of 80 kV.

Glycosaminoglycans analysis

The corneal samples were weighed and cut into small piece in 4°C environment. Immediately dehydration and degreasing by stirring in acetone and diethyl ether at room temperature for 30 min were done before dry weight of the samples was determined. GAGs were removed from proteoglycans and core proteins by stirring the samples in 0.5 N NaOH at 4°C for 15 hr and then neutralizing with 1N HCl. The mixture was heated at 100°C for 10 min to denature the protein before brought up to pH 8.0 with 1M Tris-HCl buffer. Digestion with 1.0% pronase (actinaseE; Seikagaku Kougyo, Tokyo, Japan) was performed twice at 50°C for 24 hr. Then 10% trichloroacetic acid was added into the mixture at 4°C. One hour later, the mixture was centrifuged at 1,600 g for 15 min to precipitate the suspended proteins. The supernatant was dialysed against distilled water at 4°C for 4 days. The dialysate was freeze-dried and subjected to two-dimensional electrophoresis on a cellulose acetate membrane. GAGs were stained with a solution containing 0.1% alcian blue 8GX (Merck, Darmstadt, Germany) and 0.1% acetic acid. 0.1%. GAG content was quantified by hexosamine assay according to the method of Hata³⁾.

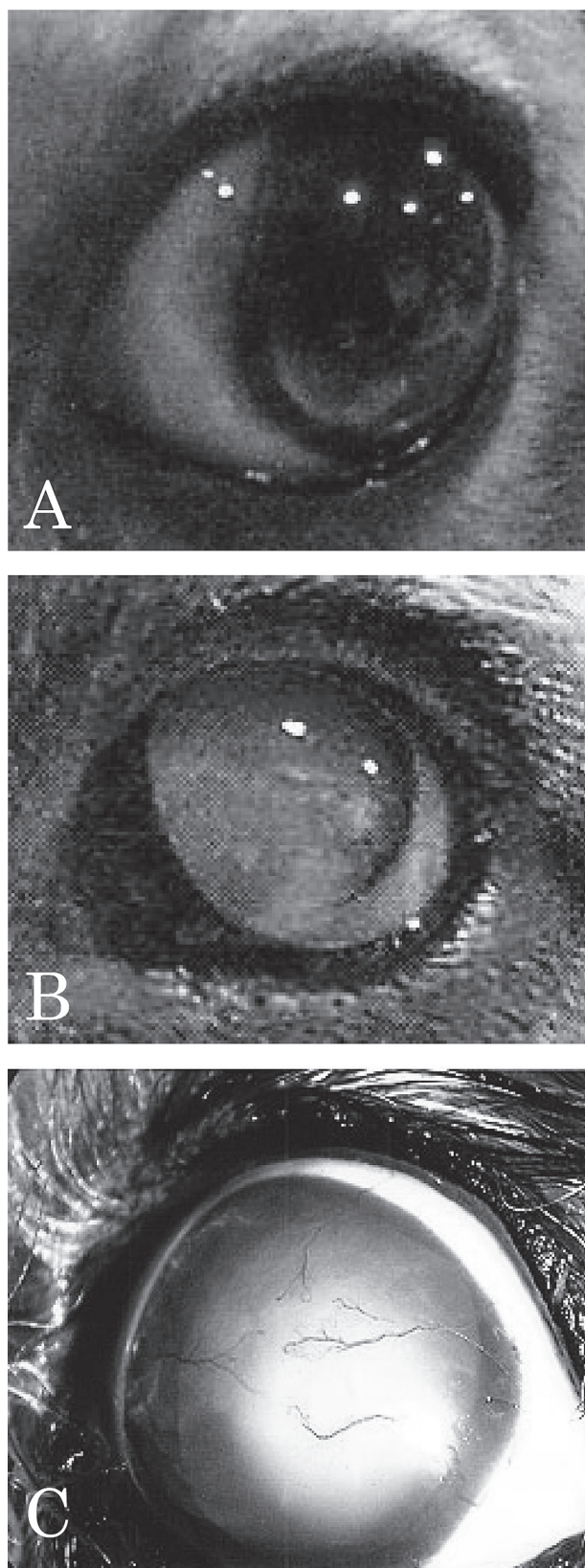


Fig. 1. Macrophoto graph showed a normal cornea (A), an opaque cornea (B) and a opaque cornea with angiogenesis (C).

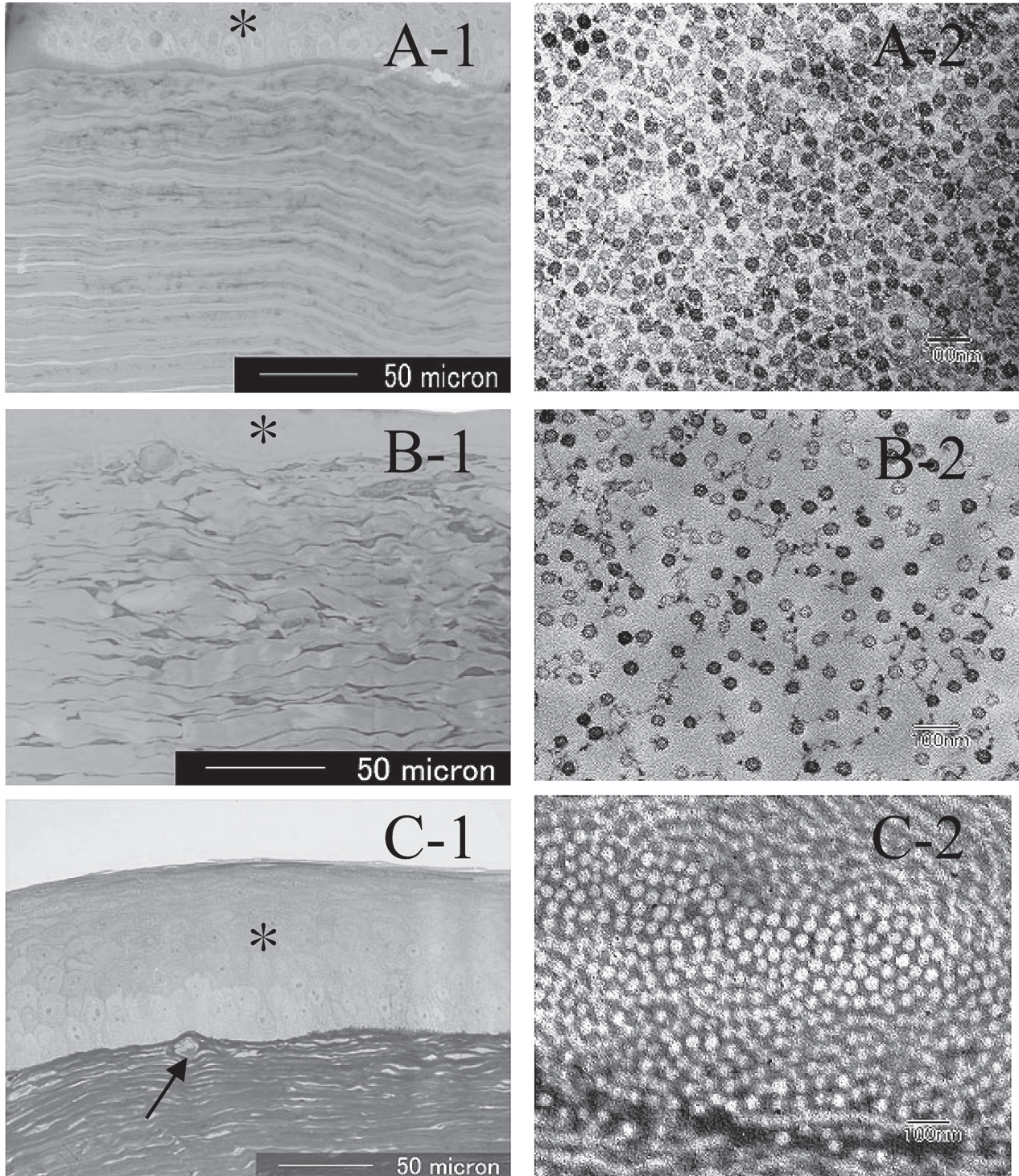


Fig. 2. Longitudinal sections of the cornea from normal (A-1), sample No.1 (B-1) and sample No.2 (C-1) observed under a light microscope. Normal arrangement of collagen fibrils was found only in normal cornea. Cross sections of the cornea from normal (A-2), sample No.1 (B-2) and sample No.2 (C-2) observed under TEM. Regular appearances of collagen fibrils and constant interfibrillar spaces were observed only in the intact cornea. The sample No.1 showed a tight arrangement with less interfibrillar spaces and abundant cellular population. Collagen fibrils running in multiple directions and irregular interfibrillar spaces were found in the sample No.2. Arrow; neovascularization, *, anterior epithelium.

Standard GAGs such as hyaluronic acid (HA), dermatan sulphate (DS), heparan sulfate (HS) and chondroitin-6-sulphate (CS) (Nacalai Tesque, Kyoto, Japan) were used as references. Proportion of average value of major GAG components was reported.

Percentage type III collagen

The samples were homogenized and incubated in 0.5 M acetic acid for 24 hr at 4°C. The precipitate removed through a centrifugation at 30,000 g for 30 min was incubated in 0.05% swine gastric pepsin (Nacalai Tesque, Kyoto, Japan) and 0.5 M acetic acid for 48 hr at 4°C. Sodium chloride at a final concentration of 0.7 M was added to the supernatant obtained after the centrifugation. The precipitate composing of type I and III collagen was resuspended in 0.5 M acetic acid. Aliquots of the samples were mixed with an equal volume of an electrophoresis sample buffer (7.2 M urea, 3.0% sodium dodecyl sulfate, 3.0% β -mercaptoethanol, 20 mM Tris hydrochloric acid pH 6.8, 2 M Tris and bromophenol blue) before boiled for 5 min. The samples were subjected to SDS-PAGE in the presence of 3.6 M urea on 5% gels and visualized with a silver staining kit (Atto Inc., Tokyo, Japan). The content of type I and III collagen was analyzed with a relevant software (NIHImage 1.61). The type III collagen content calculated as a percentage of type I plus III collagen.

Results

Normal cornea (normal) was transparent and avascular. Sample No.1 and No.2 were an opaque cornea, and No.2 was with neovascularization further (Fig. 1). Microscopic interfibrillar space in the normal was not apparent (Fig. 2A-1). Cross sectional observation of the normal was confirmed a smooth and regular arrangement of collagen fibrils in the substantia propria (Fig. 2A-2). In case of the opaque corneal samples (No.1) observed under a light microscope, collagen filamentation was indistinct and its orientation was not in a good order. Moreover, revitalization of the corneal cells was clearly seen (Fig. 2B-1). The results were consistent with a cross sectional evidence observed in Fig. 2B-2. The upper substantia propria of the opaque cornea with neovascularization (No.2) was very spectacular (Fig. 2C-1). The blood vessels were observed in just under the anterior epithelium. The sample bore indistinctive collagen fibrils with irregular running pattern. The transverse section of sample No.2 observed multidirectionally oriented collagen fibrils with different interfibrillar space (Fig. 2C-2). Its porous appearance was probably due to the abundant interfibrillar spaces.

Distribution ratio of collagen fibrils with diameters of 20, 30, 40 or 50 nm, was showed in Fig. 3. Collagen fibrils with 30 nm in diameter were about 60–98% of

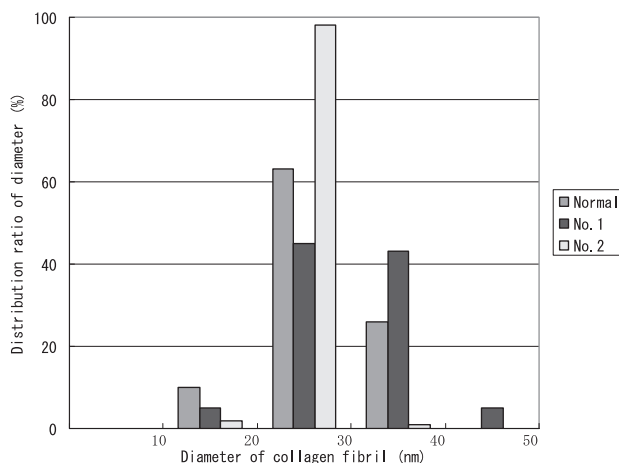


Fig. 3. The distribution ratio of collagen fibrils with different diameters. Most fibrils were designated in the 30–40 nm in diameter.

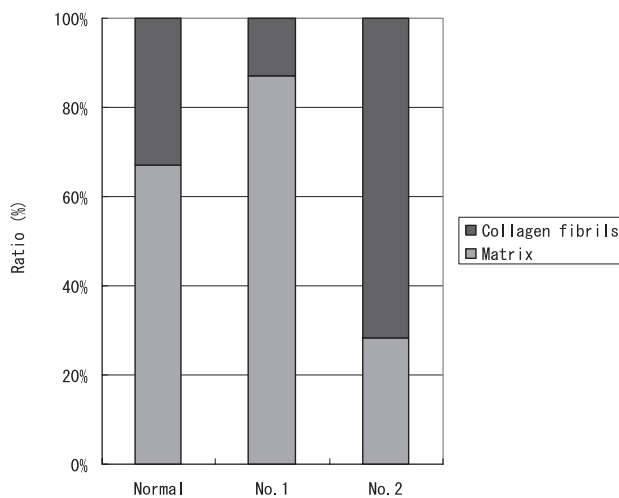


Fig. 4. The ratio of collagen fibrils and extracellular matrix. The percentage of matrix per unit of the normal, No.1 and No.2 was 67.0, 87.0 and 28.3%, respectively.

total fibril population. It was quite unfamiliar that almost all collagen fibrils of the sample No.2 had diameter restricted to the 30 nm group, while the other samples still had some distribution into various diameter range. Most fibrils of the sample No.1 were appointed into the diameter range of 30–40 nm. The average diameter of collagen fibril in the substantia propria of the normal, opaque and opaque cornea with angiogenesis was 33.2, 35.0 and 25.0 nm, respectively.

The percentage of matrix per unit area was 67% in the normal, 87% in the sample No.1 and 28.3% in the sample No.2 (Fig. 4). The matrix/collagen fibrils ratio was about

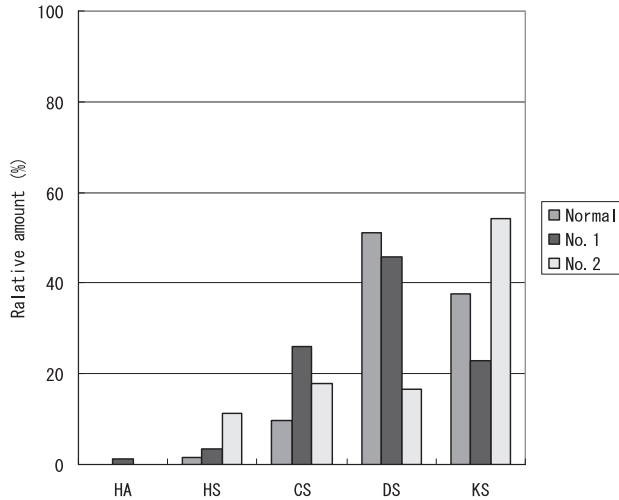


Fig. 5. The relative amount of GAGs in each sample groups. CS, DS and KS were generally found in all samples. HA was found only in the intact cornea. HA; hyaluronic acid, HS; heparan sulphate, CS; chondroitin sulphate, DS; dermatan sulphate, KS; keratan sulphate.

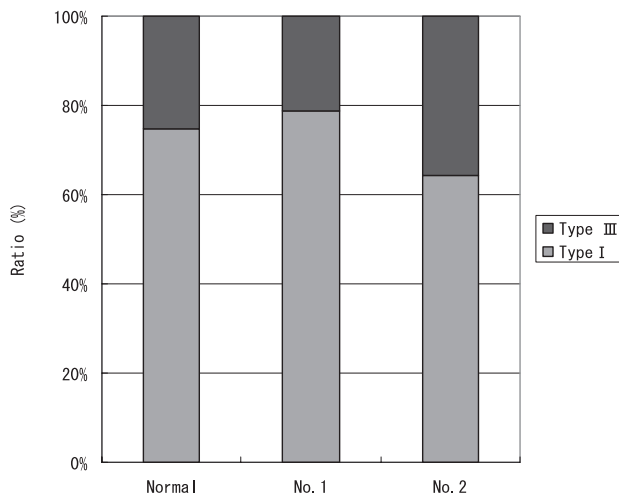


Fig. 6. The ratio of type I collagen and type III collagen. The percentage of type III collagen of the normal, No.1 and No.2 was 25.3, 21.3 and 35.8%, respectively.

two times in the normal. The ratio was varied probably due to the variation in the amount and size of collagen fibrils and amount of matrix in the opaque samples. In the sample No.1, when percentage of collagen fibrils was high, matrix contents became low. In the sample No.2, when the average diameter of collagen fibrils decreased, matrix increased. The thin collagen fibrils would arrange closely leading to the increase of the matrix.

GAGs were composed of hyaluronic acid (HA), heparan sulphate (HS), chondroitin sulphate (CS), dermatan sulphate (DS) and keratin sulphate (KS). The relative amount of HS, CS, DS and KS was 1.5, 9.7, 51.1 and 37.7% in the normal sample, 3.3, 26.0, 45.7 and 23.7% in the sample No.1 and 11.2, 18.0, 16.6 and 54.1% in the sample No.2, respectively. HA was found only in the sample No.1. Its relative amount was only 1.3% (Fig. 5).

The results obtained from an electrophoresis showed that corneal collagen was composed of type I and III collagen (Fig. 6). The ratio of the type III collagen accounted for 25.3% in the two types of collagen of the normal sample, 21.3 and 35.8% of the sample No.1 and No.2, respectively.

Discussion

Visual quality is related to a number of factors. Among these, transparency of the cornea is considered as one of the most important factor. A regular array of collagen fibrils, especially in the substantia propria of the cornea, is a critical factor to bring about the transparency of the cornea^{4, 8, 9}. When the array of collagen fibrils is altered, there certainly is some signal disturbing the normal GAG production. According to this study, changes in the relative amount of DS and KS were most remarkable. During the mature phase of the cornea, a decrease of DS and an increase of KS are synchronously because of the transparency acquisition of the cornea⁵. We speculate that the balance between these two matrix types is important to obtain normal definitive cornea. The decrease of KS in the sample No.1 could lead to a failure in maintenance of the collagen fibril diameter and interfibrillar space¹. In addition, a decrease of DS would increase the diameter of collagen fibrils^{8, 11, 12}.

Decrease of DS in the sample No.2 would cause the failure in controlling ability on the increase of fibril diameter of which production of fibril with constant diameter is promoted. Ratio of type III collagen was high in sample No.2, therefore, thin collagen fibrils such as 30 nm were abundant. Since type III collagen is increase during the convalescent stage of some injury, it would have some relationships with the neovascularization in the purpose of tissue repair. Thin collagen fibrils were also seen in agreement with increase of type III collagen ratio. Therefore, corneal opacity with a certain degree of angiogenesis seen in the sample No.2 would be caused by external factors. Biochemical and morphological properties compared between the sample No.1 and 2 were quite different probably because of having different factors. The factors involve in the sample No.1 would possibly have endogenous origins leading to the metabolic disorder of GAG. Since GAGs and collagen fibril formation have a certain degree of relationship¹¹. The imbalance of GAG production or composition would

be the cause of irregular array of collagen fibrils in the opaque cornea¹¹⁾. However, opacity of the cornea would also have other factors required for investigation further.

References

- 1) Birk DE and Trestad RL. Extracellular compartments in tendon morphogenesis: collagen fibril, bundle, and macroaggregate formation. *J Cell Biol* 1984; **99**:2024–2033.
- 2) Chikama S and Nishida T. Corneal disorder and extracellular matrix. In: *Walking of Medical Science, Extracellular Matrix, Separated Volume*, Ishiyakusyuppan, 1997; 86–90 (in Japanese).
- 3) Hata R. Microanalytical methods for glycosaminoglycans. *Protein Nucleotide Enzyme* 1973; **18**:843–857 (in Japanese).
- 4) Irk DE and Lande MA. Corneal and scleral collagen fiber formation in vitro. *Biochem Biophys Acta* 1981; **670**:362–369.
- 5) Ishii Y. Morphological and biochemical changes occurring during corneal development. *J Jpn Vet Med Assoc* 2001; **54**:843–846 (in Japanese).
- 6) Iwata S. Physiology and biochemistry of eyes. *Hirokawasyoten*, 1986; 12–28 (in Japanese).
- 7) James LF, Bruce C and Gray WC. Keratan sulfate proteoglycan during embryonic development of the chicken cornea. *Dev Biol* 1986; **116**:267–277.
- 8) Kobayashi A, Takehana K, Tajima M, Eerdunchaolu, Takahashi K and Abe M. A bovine case of dermatosparaxis characterized by dermatan sulfate deficiency. *J Jpn Vet Med Assoc* 1999; **52**:294–298 (in Japanese).
- 9) Kuboki Y, Hata R and Yoshizato M. From the mystery of the animal source to the biotechnology organ. In: *The Next Generation Protein Collagen*, Koudansya, 1991; 135–138 (in Japanese).
- 10) Ronald JM, Olga MST, Yamashita M and Vincent CH: Analysis of the proteoglycans synthesized by corneal explants from embryonic chicken. *J Biol Chem* 1989; **264**:1414–1422.
- 11) Shinkai H. Proteoglycans and glycosaminoglycans. In: *New Experimental Biochemistry*, Vol.3, 1st ed, Seikagakkai, 1991; 296–300 (in Japanese).
- 12) Takehana K, Miyata H, Abe M and Morishige T. Changes in cutaneous collagen fiber of canine dermatosparaxis. *J Jpn Vet Med Assoc* 1995; **48**:338–341 (in Japanese).