Experimental Formation of 100 nm Periodic Fibrils in the Mouse Corneal Stroma and Trabecular Meshwork

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The so-called long-spacing collagen that exhibits approximately 100 nm periodicity has been observed in the interstitial connective tissue of various organs under normal and pathological conditions. Although we, as well as many other investigators, have reported an increasing amount of these structures with age in the human trabecular meshwork, the pathological significance and the mechanism of its formation are still unknown. We incubated mouse ocular tissues in culture medium containing 20 mM ATP and prepared them for electron microscopic observation according to the method of Bruns et al (J Cell Biol 103:393, 1986). After the incubation, abundant 100 nm periodic fibrils were observed in the corneal stroma and the region of the trabecular meshwork, both of which show no structure of 100 nm periodicity under normal conditions. For the experimental formation of 100 nm periodic fibrils, ATP, acidic condition and temperature around 37°C are necessary. The 100 nm periodic fibrils observed in our experiment were very similar to long-spacing collagen, in that the dark transverse bands have 100 nm intervals and very fine filaments of 6–7 nm diameter axially connect the bands. Long-spacing collagen is not usually observed in the human cornea, even in aged persons. The results of our study suggest that the occurrence of long-spacing collagen is related to special conditions developing in the trabecular meshwork with age but not in the corneal stroma. Experimental studies of 100 nm periodic fibril formation in mice offer a useful model for the age-related increase of long-spacing collagen in the trabecular meshwork of the human eye. Invest Ophthalmol Vis Sci 30:869–874, 1989

The so-called long-spacing collagen1 is characterized by its periodicity of 100 nm or more, compared with the 64 nm periodicity of type I collagen fibrils. An occurrence of the long-spacing collagen has been reported in Descemet’s membrane of Fuchs’ corneal dystrophy,2,3 or Chandler’s syndrome,4 in the trabecular meshwork of the patient with chronic simple glaucoma,5–7 and in the extracellular matrix of lacrimal gland tumors.8 This structure is also observed in normal human tissues; it is commonly found especially in the trabecular meshwork of aged persons.8–10 The pathological significance and the mechanism of its formation are, however, still unknown.

The formation of 100 nm periodic fibrils, which resembled the long-spacing collagen mentioned above, was reported by Bruns et al.11 in human skin fibroblast culture subjected to prolonged incubation in culture medium, or in adult rat tail tendon treated with ATP. Using monoclonal antibodies against type VI collagen the same authors and Linsenmayer et al12 showed that type VI collagen is a major component of the 100 nm periodic fibrils.

We have reported an increase of long-spacing collagen with age in the human trabecular meshwork by electron microscopy.13 Long-spacing collagen, however, is not usually observed in the corneal stroma of aged persons.14 Therefore, to explain the differences in the formation of 100 nm periodic structures between the trabecular meshwork and the cornea would be important in searching for age-related changes of extracellular components in the connective tissue of the eye. The purpose of our study is to experimentally produce 100 nm periodic fibrils and to analyze the mechanism of their formation in the mouse cornea and trabecular meshwork, both of which show no 100 nm periodic fibrils under normal conditions.

Materials and Methods

Eyeballs were enucleated from adult mice (strains: dd and beige) under ether anesthesia and cut into halves in the meridional direction. Some pieces were fixed with 4% paraformaldehyde immediately after enucleation and prepared for electron microscopic observation. Other pieces were incubated in the experimental media, as detailed in Table 1. Care of animals in this investigation conformed to the ARVO Resolution on the Use of Animals in Research.
Table 1. Experimental conditions and results of 100 nm periodic fibril formation by ATP incubation

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Plus ATP experiments</th>
<th>Minus ATP controls</th>
<th>(no incubation)</th>
</tr>
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<tbody>
<tr>
<td>Medium</td>
<td>F12</td>
<td>F12</td>
<td>PBS</td>
</tr>
<tr>
<td>20 mM ATP</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pH</td>
<td>4.7</td>
<td>4.7</td>
<td>3.0</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>37</td>
<td>4</td>
<td>37</td>
</tr>
<tr>
<td>Incubation time (hr)</td>
<td>2</td>
<td>72</td>
<td>2</td>
</tr>
<tr>
<td>100 nm periodic fibril formation observed in:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cornea</td>
<td>+++</td>
<td>--</td>
<td>+++</td>
</tr>
<tr>
<td>trabecular meshwork</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Pieces of mouse eyeballs were incubated under conditions indicated in the Table. After incubation, tissues were prepared for electron microscopic observation. Presence or absence of 100 nm periodic fibrils is expressed as ++++, abundant; ++, moderately present; +, present; --, absent; NE, not examined.

As human material, an eyeball was taken at autopsy from a 79-year-old woman who had no history of ophthalmic problems other than senile cataract. Pieces of the enucleated globe were immediately fixed and processed for electron microscopy.

ATP Incubation

ATP·2Na (Sigma A-5394; Sigma Chemical Co., St. Louis, MO) was added to F12 medium (GIBCO, Grand Island, NY) or phosphate-buffered saline (PBS) at a concentration of 20 mM in experiments 1–6 (Table 1). Experiment 1 was performed by the method described by Bruns et al. In experiment 2, the incubation was carried out at 4°C for 72 hr. We also used PBS to avoid the effects of serum components (experiments 3–6). In addition, we tried a prolonged incubation of 24 hr at 37°C (experiment 4) or incubation at a low temperature of 4°C (experiment 2). As the pH decreased when ATP·2Na was added to PBS (experiments 3, 4), we neutralized the media with NaOH (experiments 5, 6).

In experiments 7–12, tissues were incubated in the media without ATP, as indicated in Table 1. When ATP was added to the media, the pH decreased to 4.7 in F12 medium (experiments 1, 2) and 3.0 in PBS (experiments 3, 4). We used media at the same pH adjusted with HCl in place of ATP to check the sole effect of an acidic condition (experiments 10–12). We measured the pH of media before and after the incubation; pH did not change during incubation period.

Electron Microscopic Observations

After incubation, each tissue was fixed with 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 2 hr, and postfixed in 1% osmium tetroxide in the same buffer for 90 min at room temperature. They were dehydrated with graded concentrations of ethanol and embedded in Quetol 812 (Nissin EM, Tokyo, Japan). Ultrathin sections were cut on a Porter-Blum MT-1 ultramicrotome using a diamond knife. The sections were stained with uranyl acetate and lead citrate and examined in a H-800 electron microscope (Hitachi, Tokyo, Japan).

Results

In the stroma of the cornea and trabecular meshwork fixed immediately after enucleation, fine filaments were dispersed in the space among periodically striated collagen fibrils of 30–50 nm diameter (Fig. 1). There were no periodic structures other than the collagen fibrils in the mouse corneal stroma. The fine filaments often exhibited beaded structures at approximately 100 nm intervals, and the diameter of the filamentous portion was 6–7 nm. They were mostly associated with the collagen fibrils. Short filaments of 6–7 nm diameter were also interspersed among the collagen fibrils. In the region of the anterior chamber angle that corresponds to human trabecular meshwork, no periodic structures like long-spacing collagens were observed, but fine filaments resembling those of the cornea were dispersed in the extracellular space where striated collagen fibrils were loosely arranged.

After 2 hr incubation at 37°C with 20 mM ATP (Exps. 1, 3), numerous fibrillar structures with dark cross-bands or nodes of about 100 nm periodicity were observed in the mouse corneal stroma (Fig. 2, Exp. 3). These fibrillar complexes, ranging in width from 25 to 150 nm, mostly paralleled the collagen fibrils. At higher magnification fine filaments of 6–7...
nm diameter axially connected the cross-bands or nodes of approximately 35 nm in axial width (Fig. 3). We tentatively call these experimentally formed fibrils “100 nm periodic fibrils (100 nm PF)” in this article. The incubation for 24 hr with ATP (Exp. 4) also produced numerous 100 nm PF. There were no morphological differences between 100 nm PF in the 2 hr and 24 hr incubations (Exp. 3 vs. Exp. 4) or in the incubations in F12 medium and PBS (Exp. 1 vs. Exp. 3) (data not shown).

In the region of the trabecular meshwork, 100 nm PF were also observed under the same conditions (Exps. 1, 3, 4). Compared with the corneal stroma, the 100 nm periodic fibrils were slenderer and shorter, and dispersed in the extracellular space of the trabecular meshwork (Exp. 1, Fig. 4). When the tissues were incubated with ATP in PBS neutralized to pH 6.6 by NaOH, no 100 nm PF were observed (Exps. 5, 6).

Incubations in the F12 medium without ATP (pH 7.4) produced no 100 nm PF in either the cornea or the trabecular meshwork (Exps. 7, 8). However, small amounts of 100 nm PF were observed in the cornea incubated in F12 medium acidified to pH 4.7 for 2 hr (Exp. 10) as well as in the PBS acidified to pH 3.1 for 24 hr (Exp. 12, Fig. 5), but not in the cornea incubated in the same PBS for 2 hr (Exp. 11). Most of the fibrils formed in these experiments were thinner than those observed in the ATP incubations, and they often showed a beaded structure. Incubations at 4°C, with (Exp. 2) or without (Exp. 9) ATP caused no 100 nm PF formation, even though the incubation was prolonged up to 72 hr.

Naturally occurring long-spacing collagen in the trabecular meshwork of a 79-year-old woman is shown in Figure 6. It has a very similar appearance to the 100 nm PF formed under experimental conditions in mice; it consists of dark periodic cross-bands, which were axially connected by fine filaments. There were, however, differences in the details of morphology. The periodicity ranged from 100 to 120 nm in the long-spacing collagen of human trabecular meshwork, as opposed to 90 to 100 nm in the 100 nm PF of mice. In the mouse cornea, the dark cross-bands were larger in axial width and more electron-dense, and the fibrils were thinner but longer and straight-running. The long-spacing collagen in the human trabecular meshwork is often associated with amorphous material that constitutes an electron-dense background.

**Discussion**

The current study showed that 100 nm periodic fibrils appear after an incubation of tissue in acidic media containing ATP at 37°C both in the cornea and trabecular meshwork of mice, where 100 nm PF are usually not found under normal conditions. We suppose that some precursors that are dispersed among the collagen fibrils aggregate to form 100 nm PF in the presence of ATP. One of the candidates for the precursors seems to be fine filaments with a beaded appearance of 100 nm periodicity. Lateral aggregation of these filaments at their beads may result in the formation of 100 nm PF. However, the amounts of these filamentous observed in the unincubated control tissues are not enough to explain the amounts of 100 nm PF formed in the experiments. Short filaments of 6-7 nm diameter interspersed among collagen fibrils may also be candidates. The
amount of all these observable precursors still seems to be smaller than that of experimentally formed 100 nm PF. There may be precursors difficult to identify unless they are artificially aggregated by an incubation with ATP. The results of Exps. 5 and 6 indicate that ATP is effective for the formation of 100 nm PF only under acidic conditions. It is suggested, therefore, that, as in the case of segment-long spacing forms aggregating from type I collagen molecules, polyanionic sites of ATP interact with surface cations of precursors, resulting in the formation of 100 nm PF.

Incubation in an ATP-free acidic medium (Exps. 10, 12) resulted in the appearance of 100 nm periodic fibrillar structures, although the amount was smaller and the fibrils were thinner than in the ATP incubations. Since the presence of ATP or similar active substances cannot be excluded from the F12 medium or corneal tissues, smaller amounts of such substances could mediate a low rate of 100 nm PF formation. In the tissue incubated at 4°C with or without ATP, the 100 nm PF were not detected within 72 hr. These results suggest that some enzymatic reactions are involved in the formation of 100 nm PF. Some proteolytic reactions might be necessary to free the precursors from their connections with other structures such as collagen fibrils.

Zimmerman et al.16 reported that type VI collagen is a major component of the extracellular matrix of the human cornea and showed by an indirect immunofluorescence technique that this collagen is distributed throughout the corneal stroma. Our result showing that the experimental formation of 100 nm PFs was remarkable in the mouse corneal stroma corresponds well to the hypothesis of Bruns et al.11 and Linsenmayer et al.12 that this periodic structure is made from type VI collagen. We assume that precursors, including type VI collagens, aggregate to form "beaded filaments" that have 100 nm periodicity, and furthermore, the beaded filaments laterally assemble to produce 100 nm periodic fibrillar structures.

We and some other investigators reported10,13 that in the human trabecular meshwork, the structures of long-spacing collagen increase with age. These banded structures are more abundantly observed in

![Fig. 6. Long-spacing collagen in the trabecular meshwork of a 79-year-old woman. Dark periodic cross-bands are connected by thin filaments. The background of the long-spacing collagen is dark with amorphous material (×59,000).](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/933377/)
the trabecular meshwork of patients with chronic simple glaucoma. On the other hand, long-spacing collagen does not usually occur in the human corneal stroma, where type VI collagens have been shown to be present. Our study has demonstrated that precursors, possibly including type VI collagens, can be aggregated into 100 nm periodic structures under suitable conditions. Special conditions causing the formation of long-spacing collagen may develop gradually with age in the stroma of the trabecular meshwork, where aqueous humor continuously permeates.

We morphologically compared the experimentally formed 100 nm PF in mice with the naturally occurring long-spacing collagen in the human trabecular meshwork. Periodic, dark cross-bands and axial fine filaments connecting the bands were observed in both cases. The periodicity ranged from 90 to 100 nm in the mouse cornea, and from 100 to 120 nm in the human trabecular meshwork. There may be some differences in size of precursor molecules between mouse and man. A standardization of specimen shrinkage, if any, will now be undertaken. The background of human long-spacing collagen in aged persons was dark with electron-dense, amorphous material that was not detected in mice. Components other than 100 nm PF will also be studied in relation to age-related changes of the extracellular matrix of the eye.

Key words: long-spacing collagen, 100 nm periodic fibrils, trabecular meshwork, corneal stroma, type VI collagen

References