Arrangement of D-Periodic Collagen Fibrils and Association of Proteoglycans with Fibrils in the Synovium of the Mouse Temporomandibular Joint

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Summary. The present study was performed to examine changes in the arrangement of D-periodic collagen fibrils in the synovium of the growing temporomandibular joint in mice. At 1 week of age, the mandibular condyle was undeveloped, and only a few collagen fibrils were recognizable in the subintimal layer of the synovium. At 8 weeks, the mandibular condyle was structurally developed with an increase of collagen fibrils in the synovium; a fully mature condyle was observed at 6 months of age. The close association of proteoglycans with collagen fibrils in the synovium of the growing joint was examined by both conventional and energy-filtering transmission electron microscopy of cupromeric blue-stained specimens. Proteoglycans were associated with D-periodic collagen fibrils in the short filamentous form in random fashion at 1 week of age, but in a regular pattern with D-periodicity at 6 months. These associations in the synovium could be correlated with the mechanical character of the temporomandibular joint.

The synovium is known to play an important role in maintaining normal joint physiology as well as in joint pathology. It consists of two types of synoviocytes and dense connective tissues; typically, cross striated collagen fibrils with a 64 nm repeating period (D-periodicity) are arranged to maintain and support the tissue structure of the synovium. Recent ultrastructural studies of synoviocytes in experimental animals have made advances (Iwanaga et al., 2000); however, the ultrastructure and arrangement of the D-periodic collagen fibrils themselves remain to be fully described in the synovium of the temporomandibular joint. We have previously reported the occurrence of type VI collagen in the synovium of the mouse temporomandibular joint (Teramoto et al., 1995); that type of collagen was abundant, especially in the intimal layer, and could be associated with D-periodic collagen fibrils via proteoglycans or glycosaminoglycans (PGs/GAGs). Elucidation of the interaction of PGs/GAGs with D-periodic collagen fibrils in the subintimal layer will give us further information concerning the functional characteristics of the synovial membrane as a whole, because the interaction of PGs/GAGs with D-periodic collagen fibrils has been confirmed in various tissues (Scott, 1988; Kimura et al., 1995; Nakamura et al., 1997).

In this study, we closely examined the ultrastructure of D-periodic collagen fibrils in the synovium, especially the subintimal layer, of the growing mouse temporomandibular joint. The association of PGs/GAGs with collagen fibrils was also studied by both conventional and energy-filtering transmission electron microscopy (TEM) of cupromeric blue-stained specimens. The mouse temporomandibular joint was used in this study because it has been intensively investigated by oral biologists, pathologists and clinicians, and useful data have been accumulated, especially in this species.

MATERIALS AND METHODS

Tissue preparation

Pieces of synovium of the temporomandibular joint were taken from mice (strain ddY, female) at 1 week, 8 weeks and 6 months of age. Three or more animals at each age were used for this study. Care of the animals in this investigation conformed to the Guide for Animal Research, Nagoya University School of Medicine.
Light and transmission electron microscopy

Tissue blocks including the synovium were immediately placed in Karnovsky's fixative for 24 h at 4°C. They were rinsed in 0.1 M phosphate buffered saline (PBS), pH 7.4, and decalcified in 10% ethylenediamine tetraacetic acid-4Na in glycerol solution, pH 7.4, at 4°C for 6 days. After washing in PBS, they were post-fixed in 1% osmium tetroxide buffered with PBS at room temperature for 90 min. They were rinsed in PBS, dehydrated in a graded series of ethanol, and embedded in Quetol 812 (Nissin EM, Tokyo). Semithin sections were stained with 1% toluidine blue in 0.1 M sodium borate for light microscopy. Ultrathin sections were cut with an ultramicrotome (Porter Blum MT-1, U. S. A.), double stained with uranyl acetate and lead citrate, and observed with a transmission electron microscope JEM 1200 (JEOL, Tokyo).

Scanning electron microscopy

Whole tissues from the temporomandibular joints were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in a 0.1 M phosphate buffer. They were immersed in a 10% aqueous solution of NaOH for 8 days at room temperature, and then rinsed in distilled water for a day (OHTANI, 1988). The specimens were processed by conductive staining with 1% OsO₄, 1% tannic acid and 1% OsO₄, for 1 h each. They were then dehydrated in a series of graded concentrations of ethanol, critical point dried (HCP-1, Hitachi critical point dryer) and coated with an osmium plasma coater (Nippon Laser Electronics, Nagoya). Observation was made under a scanning electron microscope (SEM) (Hitachi S-800, Tokyo) with an acceleration voltage of 10 kV.

Cupromeronic blue staining

Cupromeronic blue (CB) consists of electron dense precipitants of proteoglycans that scaffold PGs during precipitation, maintaining their shape and position in the tissue (SCOTT and THOMLINSON, 1998). Tissues were fixed in 2.5% glutaraldehyde/25 mM sodium acetate buffer (NaAc) at pH 5.8, followed by 3 washings in 25 mM NaAc at pH 5.8. Staining was carried out in solutions of 0.1 M MgCl₂; 25 mM NaAc at pH 5.8; 2.5% glutaraldehyde and 0.05% w/v cupromeronic blue (Seikagaku Kogyo, Tokyo) overnight at room temperature. The sections were washed in a solution identical to that in which the stain was dissolved, and stained further with 0.034 M sodium tungstate (SCOTT and THOMLINSON, 1998). They were then processed for electron microscopy.

Energy filtering-TEM observation

The energy filtering-TEM was from Hitachi (EF-1000). This microscope has been developed to improve image resolution and enhance the contrast of transmission electron microscopy images (TAYA et al., 1996); the filter consists of two sector magnetic fields, and electrons travel through the filter following γ-shape trajectories. Using this microscope, electrons in a specific range of energies can be selected and focused to produce an image. The energy filter was incorporated into a TEM for observation of thin sections without electron staining. To produce a sharp image, 100 keV-loss electrons were chosen in the present study.

RESULTS

Light microscopic profile of growing temporomandibular joint and synovial membranes

The mouse temporomandibular joint is located in front of the meatus acusticus externus (Fig. 1A). The synovium lines the inner surface of the fibrous capsule of the joint as well as most of the upper and lower articular cavities. The synovium, however, is lacking over the articulating surfaces of the temporal bone, the articular disk, and the mandibular condyle. The synovium was thin and there was little fibrous tissue in the subintimal layer (Fig. 1C, D). At 8 weeks of age, the neck was formed in the condylid process (Fig. 1E), and the fibrous tissue in the subintimal layer was fully developed (Fig. 1F, G). At 6 months, the lower articular cavity of the joint was not clear (Fig. 1H), and abundant connective tissues greatly thickened the whole synovium (Fig. 1I, J).

Arrangement of collagen fibrils in the synovial membrane

The ultrastructure of D-periodic collagen fibrils in the subintimal layer of the synovium was revealed by SEM and TEM. At 1 week of age, there were few collagen fibrils in a random arrangement (Fig. 2A, B). The spaces between the fibrils were large, containing some amorphous materials associated with collagen fibrils. At 8 weeks, collagen fibrils partially formed bundles (Fig. 2C, D). At 6 months, most of the collagen fibrils formed bundles, which piled up to
Fig. 1. Light microscopic profiles and their schematic drawings of the growing temporomandibular joint with the synovial membrane. **A.** Lateral view of the mouse skull and mandible. The *thick arrow* indicates the mandibular condyle. **B, E** and **H.** These drawings were traced from a light microscopic profile of the mandibular condyle at 1 week (B), 8 weeks (E) and 6 months (H). **C, F** and **I.** Light microscopy of the squared area in B, E and H, respectively. ×200. **D, G** and **J.** High power view of light microscopy of the synovium at 1 week (D), 8 weeks (G) and 6 months (J). *Small arrows* indicate the synovium facing the joint cavity. ×500
Fig. 2. SEM (A, C, E) and TEM (B, D, F) profiles of collagen fibrils in the synovium of the growing temporomandibular joint at 1 week (A, B), 8 weeks (C, D) and 6 months (E, F). A, C, E: ×90,000, B, D, F: ×100,000
Fig. 3. Diameter of collagen fibrils at 1 week, 8 weeks, and 6 months.

Fig. 4 A-C. Association of PGs/GAGs with D-periodic collagen fibrils in the synovium detected with CB staining. These micrographs were taken with a conventional TEM after electron staining of sections with uranyl acetate. A: 1 week, B: 8 weeks, C: 6 months. Arrows: D-periodicity of collagen fibrils. A–C: ×100,000
become collagenous layers (Fig. 2E, F). In individual bundles, collagen fibrils ran mostly in the same direction and the spaces between the fibrils were narrow. The D-periodicity of each collagen fibril was not clear at 1 week (Fig. 2B), but readily recognizable at 8 weeks (Fig. 2D) and 6 months (Fig. 2F).

The thickness of collagen fibrils in the synovium was measured in thin sections (Fig. 3). Very thin fibrils about 20 nm in thickness were observed at 1 week. At 8 weeks, some fibrils were thicker, though the thin fibrils were still interspersed. Large values of standard deviation indicated that there were considerable differences in fibril thickness. At 6 months of age, almost all fibrils had a uniform thickness of about 40 nm.
Detection of the association of proteoglycans with D-periodic collagen fibrils by cupromeric blue staining

Cupromeric blue (CB) effectively stains proteoglycans associated with collagen fibrils. The CB-stained subintimal layer of the synovium was observed by TEM with light electron staining of uranyl acetate. There were many CB stained fine filaments, which we call proteoglycan (PG)-filaments, on the surface of collagen fibrils at 1 week (Fig. 4A), 8 weeks (Fig. 4B) and 6 months (Fig. 4C). The association pattern of PG-filaments on the D-periodic collagen fibrils seemed regular at 6 months (Fig. 4C). Moreover, the D-periodicity of each fibril was clearly observed at 8 weeks (Fig. 4B) and 6 months (Fig. 4C). This indicates both CB and uranyl acetate stained GAGs being directly associated with collagen molecules at 8 weeks and 6 months, resulting in a clear banding pattern of the fibrils. At 1 week the striation was faint (Fig. 4A), suggesting that low amounts of GAGs could be associated with the collagen molecules in the young synovium.

The PG-filaments and the D-periodicity of the collagen fibrils were unclear even at 8 weeks and 6 months when CB staining was not performed (Fig. 2B, D, F).

Analysis of PG-filaments with energy filtering TEM

Since electron staining with uranyl acetate on CB stained fibrils is sometimes faint in part of the PG-filaments, we observed the CB-stained specimens by energy filtering TEM without electron staining. With this method, all PG-filaments associated with collagen fibrils could be demonstrated (Fig. 5A–C). The association of PG-filaments with D-periodic collagen fibrils was not uniform at 1 week. At 6 months, PG-filaments were associated regularly with the long axis of collagen fibrils at similar intervals of about 65 nm. In the growth process from 1 week to 6 months, the PG-filaments became shorter (Table 1) and thinner.

<table>
<thead>
<tr>
<th>Time</th>
<th>Length (nm)</th>
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<tbody>
<tr>
<td>1 week</td>
<td>114.00±20 (n=56)</td>
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<tr>
<td>8 weeks</td>
<td>94.56±19 (n=74)</td>
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<tr>
<td>6 months</td>
<td>90.80±14 (n=49)</td>
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DISCUSSION

In the present study, both the ultrastructural arrangement and association of PGs with D-periodic collagen fibrils were closely studied in the synovium of the growing temporomandibular joint of mice. Developmentally, the mice at 1 week after birth were still nursing, and weaning and feeding had not begun at this time. At 8 weeks after birth, the mice had grown considerably, and the joint exhibited a mature appearance. At 6 months of age, the body structures of mice were fully mature with signs of aging; atrophy of condylar surface was sometimes observed. It is thus reasonable that collagen fibrils in the 1 week synovium were not developed. Changes in the amounts and thickness of collagen fibrils during the development may be concerned with the resistance against mechanical stress.

The co-occurrence of PGs/GAGs and collagen fibrils implies an interaction between them (Scott 1988; Scott and Thomlinson, 1998) which may correlate to the functional characteristics of the temporomandibular joint. The length and width of PG-filaments differed at each of the three ages. This indicates that PG-filaments contribute toward maintaining spaces between D-periodic collagen fibrils, thus playing an important role in regulating the arrangement of collagen fibrils. We have previously reported that type VI collagen is abundant in the synovium (Teramoto et al., 1995). Type VI collagen may interact both with D-periodic collagen fibrils and with PGs. In the sclera, PGs were located on the surface of D-periodic collagen fibrils, chiefly on the d (XI) and e (IX, X) bands and interfibrillar type VI collagens (Kimura et al., 1995). Although the collagen bands with which PGs associate were not clarified in the present study, the combination of D-periodic collagen fibrils, type VI collagens, and PGs is probably closely related to the physical characteristics of the temporomandibular joint synovium.

In the present study, an energy-filtering TEM was also used to detect PGs/GAGs associated with the D-periodic collagen fibrils in CB-stained specimens. Since the image contrast is enhanced by this microscope, we succeeded in observing the PG-filaments without electron staining. Electron staining with uranyl acetate and observation with conventional TEM sometimes lead to difficulties with the detection of PGs/GAGs in CB-stained tissues. Thus, the energy-filtering TEM is useful for more precise and accurate observations of histochemically stained tissues than with the conventional TEM.

In conclusion, we have documented in detail the
ultrastructure of collagen fibrils and PG-filaments in the synovium of the mouse growing temporomandibular joint. Since the synovial collagen fibrils probably play an important role in maintaining joint physiology, this study provides basic knowledge for the morphological analysis of joint diseases such as osteoarthritis as well as for the effect of aging in the temporomandibular joint.

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REFERENCES


