Histol Histopathol (2004) 19: 1033-1041

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Ultrastructural study of upper surface layer in rat mandibular condylar cartilage by quick-freezing method

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Summary. The purpose of the present study is to clarify native ultrastructures of upper surface layers of the rat mandibular condylar cartilage in vivo by a quick-freezing method. The mandibular cartilaginous tissues were removed with their articular discs attached without opening the lower joint cavity. The specimens were processed for light microscopy, transmission or scanning electron microscopy. Deep-etching replica membranes were also prepared after the routine quick-freezing method. The upper surface layer was well preserved by the quick-freezing method. The cartilaginous tissues, which were fixed without opening their articular discs, appeared to keep better morphology than those after opening them. The upper surface layer was thicker than the corresponding layer as reported before. It consisted of atypical extracellular matrices with lots of apparently amorphous components, which were distributed over typical collagen fibrils, by conventional electron microscopy. As revealed with the replica membranes, it also consisted of variously sized filaments and tiny granular components localized on the typical collagen fibrils. A pair of stereo-replica electron micrographs three-dimensionally showed compact filaments within the upper surface layer. The quick-freezing method was useful for keeping native ultrastructures of the fragile upper surface layer in the mandibular condylar cartilage, which may be functionally important to facilitate smooth movement of the temporomandibular joint.

Key words: Mandibular condylar cartilage, Upper surface layer, Quick-freezing, Freeze-drying, Deep-etching

Introduction

The articular cartilage of animals is a specialized biological tissue, which consists of reticular collagen fibers and also contains lots of proteoglycans and water (Silva, 1971; Wilson, 1978; Gardner et al., 1983; Wilson and Gardner, 1984; Joseph and Lawrence, 1988; Riesle et al., 1998). Functionally it plays an important role in withstanding compressive loading forces and maintaining smooth movement of articular joints (Ali et al., 1993; Lang et al., 1993; Chen and Broom, 1998; Tanaka et al., 1998). For such a unique long-lasting function of the general joints, the upper surface layer of the articular cartilage must be essential for facilitating its lubrication for the smooth movement (Hills and Monds, 1998a,b; Hills and Thomas, 1998; Schwartz and Hills, 1998).

It is well-known that a temporomandibular joint (TMJ) displays some unique characteristics among various types of joints, which are different from those in hip or knee joints. The TMJ can functionally rotate, but also widely glide each time, which is essential for complicated jaw movement during mastication (Wilson, 1978; Pirttiniemi et al., 1996). Therefore, the TMJ receives strong mechanical compression forces, which occur continuously during the biting movement (Gardner et al., 1983, Wilson and Gardner, 1984). Such functional characteristics of the TMJ let us speculate that unique ultrastructures in vivo might exist within the upper surface layer of the TMJ cartilage in addition to articular fluid (Ueki et al., 1989).

The ultrastructure of the articular cartilage surface was already examined on ultrathin sections by conventional electron microscopy (Nishida et al., 1995; Orford and Gardner, 1985). On the other hand, the upper surface layer in condylar cartilages of TMJ has also been examined by the conventional electron microscopy, which always needs chemical fixation and dehydration steps during preparation procedures (Appleton, 1975, 1978; Inoue et al., 1969; Mizuno et al., 1990; Segawa and Takiguchi, 1992). These conventional studies reveal some ultrastructural features of the mandibular condylar cartilage as follows. The TMJ cartilage consists mainly of typical fibrous cartilage even at the superficial layer, while almost other types of joint surface cartilages were classified as the hyaline cartilage, which are similar to those of hip or knee joints. Each layer within the TMJ is

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reported to show regional variations of morphology even on the whole condylar cartilage. They, however, demonstrate no clear evidence for the upper surface layer with articular fluid of the mandibular cartilage in situ (Wilson and Gardner, 1984; Ueki et al., 1989). Some other researchers also reported the morphology of the upper surface layer using conventional electron microscopic techniques (Asari et al., 1994; Hills and Monds, 1998a,b), but their findings obtained by such various conventional methods appear to be in variation without clearly convincing demonstration for the condylar superficial layer. Therefore, there has not been widely accepted consensus about the native ultrastructure of the mandibular condylar surface layer until now (Hills and Thomas, 1998).

One of the considerable reasons, why the previous studies hardly clarify the native ultrastructure of the surface cartilage layer in situ, may be due to fragility of its surface structural components (Hills, 1989). We have already examined rat knee joints using a quick-freezing method, which is one of the best morphological approaches to maintain native ultrastructures of fragile cartilaginous tissues and cells, including the superficial amorphous layer of the hyaline cartilages at hip or knee joints (Toriumi et al., 1996; Leng et al., 1998; Watanabe et al., 2000). Recently, we also demonstrated that both hyaluronic acid and fibronectin are immunohistochemically localized as a double layer in the upper surface layer of rat TMJ cartilages in vivo (Zea-Aragon et al., 2004). In the present study, we applied the quick-freezing method for examining the upper surface layer in situ of the rat TMJ cartilages.

Materials and methods

Forty-five male Wistar rats, aging 8-12 weeks and weighing between 200 and 250 g, were used for the present study. They were divided into three groups under different preparation procedures for light microscopy (LM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM), as described below. The quick-freezing method was also used for both TEM and SEM specimens. The present experiment, as reported before (Toriumi et al., 1996; Leng et al., 1998; Watanabe et al., 2000), was performed in accordance with the guidelines governing animal experiments within the University of Yamanashi.

Light Microscopy

Under anesthesia with sodium pentobarbital, five rats were perfused via hearts with 2% paraformaldehyde (PF) in 0.1 M phosphate buffer (PB), pH 7.4, for about 5 minutes. Mandibular condylar cartilages, keeping their closed lower joint cavities, were resected and additionally fixed with 2% PF in PB at 4 °C overnight. Some specimens were decalcified with 5% ethylenediaminetetraacetic acid (EDTA) for one week, routinely dehydrated in a graded series of ethanol (50%, 70%, 80%, 90%, 95% and 100%) at room temperature, and then embedded in paraffin wax. They were cut into 5µm-thick sections with a microtome.

For the cryostat section preparation, other PF-fixed specimens were immersed with 20% glycerol and 10% sucrose in PB at 4 °C after the fixation with 2% PF in PB, embedded in OCT compound (Sakura Finetechnical Co., Tokyo, Japan), and subsequently frozen in liquid nitrogen (-196 °C). The frozen specimens were cut into 5µm-thick sections by a cryostat machine cooled down at -30 °C. Both paraffin sections and cryosections were mounted on slide glasses and stained with hematoxylin-eosin (HE), alcian blue (pH 2.5), or sudan black for lipids in the routine staining manners. They were finally observed under a light microscope.

Transmission electron microscopy

Under anesthesia with sodium pentobarbital, five male Wistar rats were perfused via hearts with 2% PF in PB, which were followed by 2.5% glutaraldehyde (GL) in PB for about 5 minutes. The mandibular condylar cartilaginous tissues were divided into two subgroups due to the different preparation procedures to evaluate the degree of morphological preservation revealed by each procedure. First, the mandibular condyles were resected from the temporomandibular joint without their articular discs. They were subsequently re-fixed with 2.5% GL in PB at 4 °C for 1 hour and were postfixed with 1% osmium tetroxide in PB. After washing with PB and routine dehydration in a graded series of ethanol, they were embedded in epoxy resin, Quetol 812 (Nisshin EM Co., Tokyo, Japan). Ultrathin sections were cut at 80 µm thickness and doubly stained with uranyl acetate for 10 minutes at room temperature and lead citrate for 2 minutes. They were evaporated with carbon and observed in H-600 and H-7500 electron microscopes (Hitachi Co., Ibaraki, Japan) at an accelerating voltage of 80 kV. Second, the mandibular condyles with intact articular discs attached were removed from the temporomandibular joint, fixed with 2.5%GL in PB and subsequently prepared for TEM in the similar manners as described above.

Scanning electron microscopy

Under anesthesia with sodium pentobarbital, five rats were perfused with 2.5% GL in PB under the same conditions as described in the previous section. The mandibular condyles were resected with or without intact articular discs, as referred to as a “non-open”or “open” lower joint cavity, respectively. The articular disc for the non-open lower joint cavity was slightly cut a few times with razor blades to penetrate the fixative fully into the articular cavity. The cartilaginous tissues of both groups were then fixed again with 2.5% GL in PB at 4 °C for 1 hour, washed in PB several times, and postfixed with 1% osmium tetroxide in PB for 1 hour. After the double fixation, they were quickly frozen in an

isopentane-propane mixture (-193 °C) cooled in liquid nitrogen (Toriumi et al., 1996). The frozen cartilaginous surface tissues were freeze-fractured with a scalpel in liquid nitrogen and freeze-dried with t-butyl alcohol for SEM. The specimens were ion-sputtered with platinum and palladium with a thickness of 12nm, and observed in an S-4500 scanning electron microscope at an accelerating voltage of 5 kV(Hitachi Co., Ibaraki, Japan).

Quick-freezing and deep-etching method

The quick-freezing and deep-etching (QF-DE) method was also performed as described below. Under anesthesia with sodium pentobarbital, other three rats were also perfused with 2% PF in PB via hearts. The mandibular condyles with intact articular discs were resected from the temporomandibular joint. Then the articular discs were cut open by razor blades and immediately frozen in the isopentane-propane mixture (-193 °C) cooled in liquid nitrogen, as reported before (Toriumi et al., 1996). The frozen upper cartilage surface tissues were freeze-fractured with a scalpel in liquid nitrogen and deeply etched in Eiko FD-3AS apparatus (Eiko Co., Tokyo, Japan) under vacuum conditions, 2-$4x10^{-7}$ Torr, at -95 ºC for 15-20 minutes. After the deep-etching, the specimens on a cooled stage in FD-3AS were rotary-shadowed with platinum up to a total thickness of about 2 nm and additionally with carbon, as reported before (Toriumi et al., 1996). The QF-DE replicas with the specimens were floated on household bleach solution to dissolve the cartilaginous tissue components. The cleaned replica membranes were picked up onto Formvar-filmed grids and observed in a Hitachi H-7500 transmission electron microscope at an accelerating voltage of 80 kV. Electron micrographs were printed from invented negative films to make the background look bright. In addition, stereo-paired replica electron micrographs were also taken at tilting angles of ±5˚ to get three-dimensional images of the upper surface layer of the mandibular condylar cartilage.

Results

Light microscopy

Figure 1 shows light micrographs of the upper surface layer of the condylar cartilage obtained from conventional paraffin sections (Fig. 1a,c,e) after the routine chemical fixation and dehydration, and also other

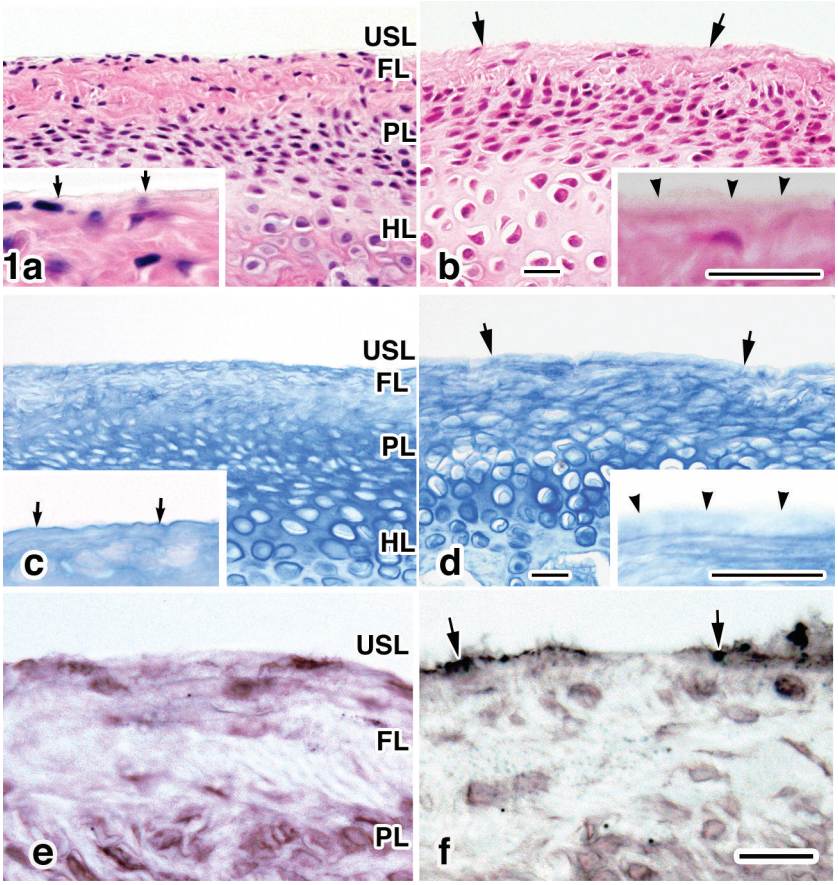

Fig. 1. Light microscopic views of the articular cartilage of the temporomandibular joints of specimens without articular discs. **a, c, e.** Paraffin sections. **b, d, f.** Cryostat sections. **a-b.** Hematoxylin-eosin (HE) staining. **c-d.** Alcian blue staining. **e-f.** Sudan black staining. Note thin ambiguous superficial layers in the paraffin sections (**a and c**, inset arrows), in comparison with thick superficial layers (arrows, about 10-20μm in depth) in the cryostat sections, (**b and d**, inset arrowheads). USL: upper surface layer; FL: fibrous layer; PL: proliferative layer; HL: hypertrophic layer. Scale bar: 20 μm.

cryostat sections (Fig. 1b,d,f), which were stained with hematoxylin-eosin (HE) (Fig. 1a,b), alcian blue (Fig. 1c,d) and sudan black (Fig. 1e,f). It should be notified that all of these sections were prepared without opening the articular discs. In the paraffin sections (Fig. 1a,c,e), thin superficial layers were observed on the articular surface cartilage. In contrast, relatively thicker superficial layers (about 10-20 μm in depth), which were heavily stained with alcian blue and less stained with HE, were detected on the articular cartilage in the cryostat sections (Fig. 1b,d). Another sudan black stain, which is a specific dye for lipid component, positively showed lots of dark contents within the upper surface cartilage layer (Fig. 1f), only when the condylar cartilage tissues were prepared by a cryostat. As shown in Figure 1e, however, the cartilage specimens prepared with routine chemical fixation and dehydration steps, which were allowed to dissolve lipid components, failed to preserve the upper surface layer darkly stained with sudan black.

Transmission electron microscopy

Figure 2 shows electron micrographs of the condylar upper surface layer in the articular cartilage obtained by the conventional electron microscopic procedures. To determine the degree of ultrastructural preservation produced by different preparation procedures, the articular surface cartilage tissues prepared with their intact articular discs (Fig. 2a) were compared with those specimens prepared after opening them (Fig. 2b). The cartilaginous specimens without articular discs possessed extremely uneven upper surface layers (Fig. 2b), which appeared to be partially destroyed and lost due to mechanical damages and chemical modification during the preparation steps. The superficial layer maintained, therefore, less amounts of rough and residual components existing over them (Fig. 2b, inset). To the contrary, the articular cartilage specimens prepared with keeping the intact articular discs showed mostly smooth upper surface cartilage tissues covered with abundant amorphous component layers (Fig. 2a). Many collagen fibrils, running in parallel organization toward horizontal direction, were clearly seen just beneath the upper surface layer. A higher-magnified view of the upper surface layer revealed heterogeneously electron-dense architectures within the thick amorphous layer, which could be easily detected in the articular

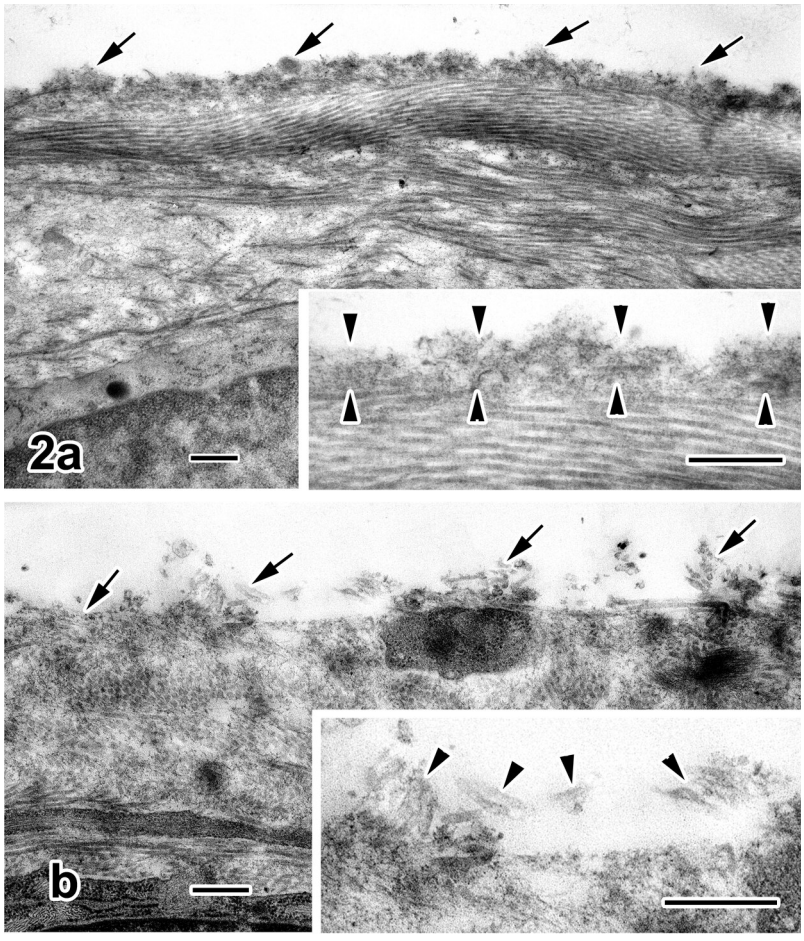

Fig. 2. Conventional electron micrographs of the articular cartilage of rat temporomandibular joints. Taken at an accelerating voltage of 80kV. **a.** A specimen prepared by the "non-open disc" technique. **b.** A specimen prepared after opening the articular disc. Note that the specimen treated without the articular disc possesses uneven or thin upper surface layers (**b**, arrows) and also that collagen fibrils are seen to be exposed **(b)**, in comparison with thicker superficial amorphous layers (**a**, arrows) in the "non-open" specimens **(a)**. **Insets:** higher magnification. Thick upper surface layers are indicated between two arrowheads **(a)**, but they are easily lost (**b**, arrowheads). USL; upper surface layer. Scale bar: 0.5 μm.

cartilage specimens prepared with the non-open articular discs (Fig. 2a, inset). The upper surface layer consisted of relatively electron-dense components, which appeared to be attached onto thin filamentous components. No cellular components were seen within the upper surface layer.

Scanning electron microscopy

To get three-dimensional images of the upper surface layer, the condylar cartilaginous tissues prepared with the quick-freezing were also examined by scanning electron microscopy. The condylar cartilaginous tissues with the intact articular discs showed definitely different ultrastructures of the upper surface layer (Fig. 3a). The uppermost region, which was considered to keep its native morphology with non-open articular capsules, possessed additional compact and amorphous materials onto the superficial layer of the condylar cartilage. Typical thick fibrous bundles were rarely seen in the amorphous structure, but filamentous networks were always detected there.

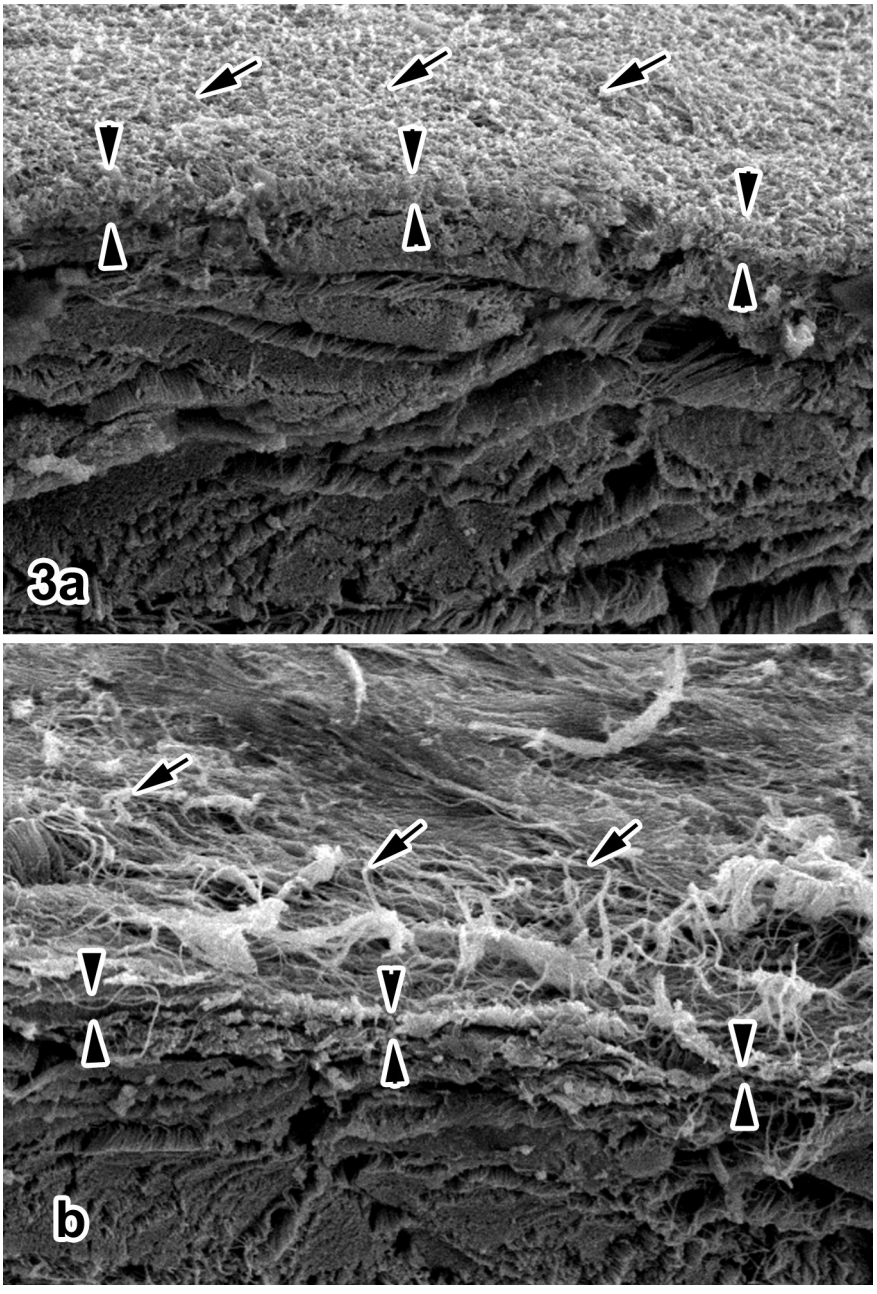

Fig. 3. Scanning electron micrographs of the articular cartilage of rat temporomandibular joints prepared by the quick-freezing method. Taken at an accelerating voltage of 5.0 kV**. a.** A "non-open" capsule specimen. **b.** An "open" capsule specimen. **a.** The upper surface layer is localized over the collagen meshwork (FL) along the condylar surface, as indicated between two arrowheads. The "non-open" articular cartilaginous tissue shows compact and aggregated filamentous networks in the superficial region (arrows). **b.** In contrast, the "open" capsule specimens possessed rough and fibrous components in the upper surface layer with collagen fibrils exposed (arrows). Also a thinner and not well defined upper surface layer is observed between two arrowheads. USL: upper surface layer; FL: fibrous layer. Scale bar: 1 μm.

The cartilage specimens, which were prepared after opening the articular capsules, possessed rough and fibrous matricial components of the upper surface layer (Fig. 3b). The uppermost contour of the surface layer was irregular with elevation and depression, and some thick fibrous deposits were aggregated onto the underlying fibrous structures. Such ultrastructural features of the upper surface layer resembled the superficial layer images, which had been previously reported with the conventional electron microscopic examination (Appleton, 1978; Toller and Wilcox, 1978).

Quick-freezing and deep-etching method

Three-dimensional *in situ* architectures of the upper surface layer were visualized at high-power resolution by the QF-DE method. The upper surface layer contained networks of variously sized filamentous structures (Fig. 4). Many tiny granular materials were also seen to be attached onto the filaments. Higher-magnified views of the same region revealed the relationship between filaments and granular components in detail (Fig. 4, lower right inset). The upper surface

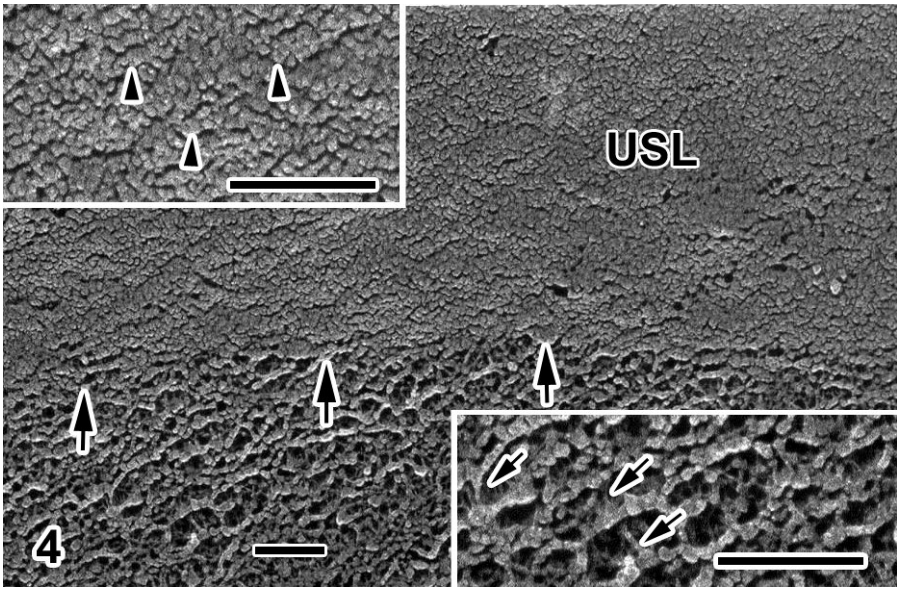

Fig. 4. Replica electron micrographs of the upper surface layer of rat temporomandibular joints prepared by the quick-freezing and deep-etching method. Taken at an accelerating voltage of 80 kV. The most superficial region of the upper surface layer (USL) is observed and freeze-fractured (arrows) in the liquid nitrogen during the preparation procedure. The superficial region appears to be flat and composed of compactly attached granular components (upper left inset, arrowheads). The deeper parts of the superficial region consist of filamentous networks and tiny granular components (small arrows). USL; upper surface layer. Scale bar: 0.2 μm.

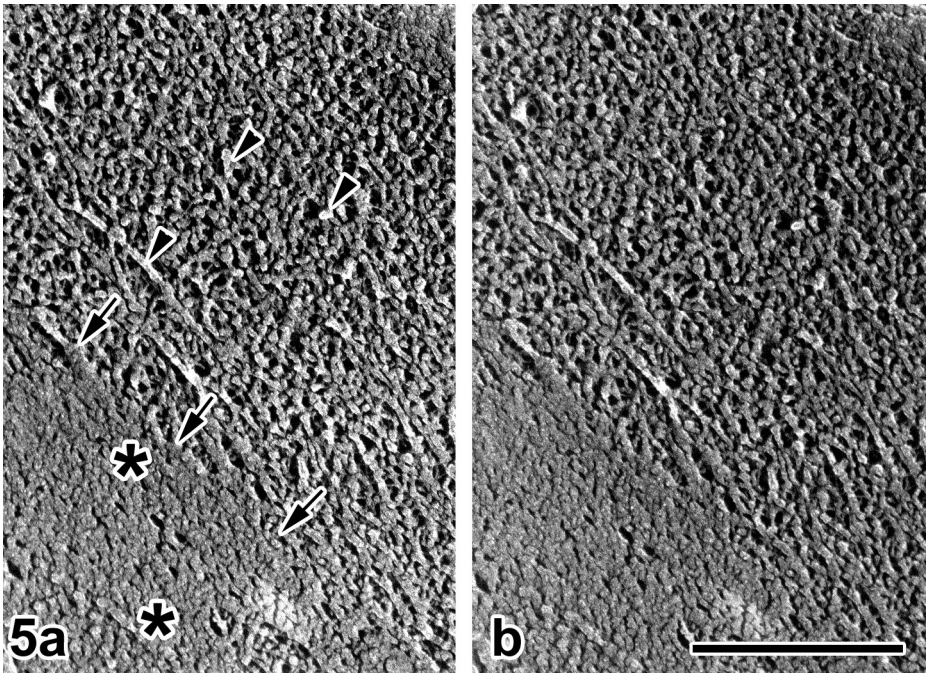

Fig. 5. Stereo-paired replica electron micrographs of the upper surface layer of the articular cartilage in rat temporomandibular joints. Taken at an accelerating voltage of 80 kV with tilting angles of ±5°. The superficial regions (asterisks) and the freeze-fractured tissue edges (arrows) are three-dimensionally shown. The slightly deeper part of the superficial region shows filaments and tiny granular components (arrowheads). Scale bar: 0.2 μm.

layer possessed variously sized networks, which were mixed with short filaments and tiny granular components attached onto them. In addition, the upper surface layer presented an uppermost smooth contour with compact granular components (Fig. 4, upper left inset)

The stereo-paired replica electron micrographs demonstrated a clear three-dimensional view of the upper surface layer architecture (Fig. 5). The uppermost surface regions exposed to the articular cavity possessed compact granular components and some fine network architectures, which included thinner filaments in comparison with those in deeper regions (Fig. 5a,b).

Discussion

In the present study, using various preparation protocols especially for electron microscopy, we determined the degree of morphological preservation of the upper surface layers of rat temporomandibular joint (TMJ) cartilage. The cryosections, which were prepared by a cryostat machine following the freezing method, maintained the upper surface layers in the condylar cartilage thicker than those chemically fixed, dehydrated and routinely embedded in the paraffin wax. The cartilaginous tissue preparations with the conventional chemical fixation often lost their soluble extracellular matrices, which could be easily dissolved into some chemical solution (Toriumi et al., 1996; Leng et al., 1998). Therefore, soluble amorphous components were easily removed and lost in the articular cartilage, but the modified surface regions still contained stiff matricial collagen fibrils alone (Meachim and Roy, 1969; Wilson, 1978).

The present preparation technique for articular cartilages of TMJ without opening their articular discs was useful for maintenance of native morphology of the upper surface layer in the articular cartilages. The cartilage specimens with the closed articular disc possessed thicker upper surface layers, as compared with those with the open articular disc (Buckwalter and Rosenberg, 1988; Ali et al., 1993). There might be various kinds of chemical or mechanical effects on the cartilage samples during the preparation steps for electron microscopy (Grodzinsky et al., 2000). Such effects might also occur during the resection step of TMJ before the fixation and dehydration steps. The SEM images obtained from the cartilage specimens with the open articular capsules possessed exposed collagenous fibrils without the upper smooth amorphous layer (Ali et al., 1993). The protective coverage with the TMJ disc definitely improved better morphological preservation of the upper surface layer on the TMJ cartilage. Other previous morphological studies may pay little attention for keeping the in situ ultrastructures of the upper surface layers, because of loosing the protection of the articular discs (Silva, 1971; Wilson, 1978). More native ultrastructures of the upper surface layers were obtained by our present preparation techniques with "non-open" articular capsules.

In addition, the quick-freezing method was useful for keeping three-dimensional ultrastructures of the upper surface layer in situ. The QF-DE method has already clarified compact architectures of filaments and granular components in the amorphous surface layer of articular cartilages of rat hip or knee joints (Leng et al., 1998; Watanabe et al., 2000). It is essential to understand functional meanings of the upper surface layer of articular cartilages in TMJ. In the present study, we obtained better images of the upper surface layer by the QF-DE method. Our examination on such specimens suggested that it was an excellent method to visualize their native ultrastructures. The QF-DE method demonstrated that the upper surface layer consisted of short filaments and tiny granular components attached onto them. Such an ultrastructure of the upper surface layer is a little different from that of rat knee or hip joints, as already revealed by the similar preparation method (Watanabe et al., 2000). The amorphous surface layer on the hip or knee joints is relatively simple in comparison with that on the TMJ cartilage (Toriumi et al., 1996; Watanabe et al., 2000). The ultrastructural features of the upper surface layer of the TMJ resembled some three-dimensional filamentous networks of proteoglycans revealed by the QF-DE method (Toriumi et al., 1996; Leng et al., 1998). In those studies, cartilaginous matrices in the rat hip joints consist of fine reticular networks, suggesting the existence of proteoglycans, as already reported (Ueda et al., 1997, 2001; Joseph and Lawrence, 1988). The network architectures of proteoglycans would be reasonable to reinforce and preserve the articular cartilage during the TMJ movement (Tajima et al., 1998; Shibata et al., 2001).

To understand the TMJ function, we need to know what kinds of components exist in the upper surface layer of the TMJ cartilage (Ghadially et al., 1965). A suggestion for the components was obtained from the present light microscopic observation with sudan black staining. The residual upper surface layer of the articular cartilage was heavily stained with the sudan black, indicating that it contained lipid components, as reported before (Ghadially et al., 1965). We have already examined the surface layers of the articular cartilage in rat hip or knee joints by using our similar cryofixation techniques (Watanabe et al., 2000). In the previous study, many granular lipid-like structures were detected within the surface amorphous region, probably corresponding to the upper surface layer of the TMJ cartilage (Leng et al., 1998; Watanabe et al., 2000). The similar structures of the rat TMJ cartilage are recently reported (Grodzinsky et al., 2000), and they are also detected on human TMJ cartilages (Rahamim et al., 2001). In the present study, our SEM observation showed filamentous networks on the TMJ articular cartilage, and also the TEM images obtained from the replica specimens showed tiny granular structures in the upper surface layer. They may represent tiny lipid

structures, corresponding to the lipid-like granules on the rat hip or knee joints (Watanabe et al., 2000). The compact filamentous networks with granular components in the upper surface layer are already revealed by the QF-DE method, supporting this idea (Leng et al., 1998; Watanabe et al., 2000). The TMJ probably shows specialized morpho-functional features, being different from those in the hip or knee joints. Recently, we demonstrated that both hyaluronic acid and fibronectin are immunohistochemically localized as a double layer in the upper surface layer of the rat TMJ cartilages (Zea-Aragon et al., 2004). The existence of such components in the lipid-containing surface regions on the TMJ cartilage is a little different from those in other movable hip or knee joints, as reported before (Ueki et al., 1989; Akisaka et al., 1998).

The articular cartilage of TMJ plays an important role in withstanding compressive loading forces and maintaining smooth movement of the TMJ. For such a function, the upper surface layer of the TMJ cartilage must be useful for facilitating the smooth movement during mastication (Asari et al., 1994; Poikela et al., 2000). Moreover, the TMJ does not only rotate but also glide for relatively long distance for coordinating movement of the jaw during the mastication (Wilson, 1978; Werner et al., 1991; Pirttiniemi et al., 1996). The TMJ also supports each biting force during the similar mastication (Gardner et al., 1983; Wilson and Gardner, 1984; Grodzinsky et al., 2000; Van den Lest et al., 2000). The present study demonstrated that the upper surface layer of the articular cartilage was much thicker than the corresponding layer previously described in other morphological studies by conventional electron microscopy with the routine preparation techniques (Orford and Gardner, 1985; Nishida et al., 1995). It is reasonable to propose that the thick surface layer is functionally important for the smooth movement of TMJ.

The upper surface layer of the TMJ cartilage was found to consist of the mixture of filamentous networks and tiny granular components. Its filamentous ultrastructure is similar to that of typical extracellular matrices, which play some roles in supporting the architectures of such dynamic tissues as glomerular basement membranes (Ohno et al., 1996) and holding biological substances in the cartilaginous tissues (Ueda et al., 1997, 2001; Leng et al., 1998; Watanabe et al., 2000). It is concluded that the upper surface layer of the TMJ cartilage has important functions to support loading mechanical forces and facilitate smooth movement of the TMJ during mastication (Ali et al., 1993), which might be compatible with its ultrastructural features.

Acknowledgements. The present study was supported in part by a Grant-in-aid from the Japanese Ministry of Education, Science, Sports and Culture. The authors thank Dr. I. Takayama, Department of Anatomy, Faculty of Medicine, University of Yamanashi, Japan, for his useful advice, and also Dr. Y. Fujii, Miss Y. Kato and Mr. Y. Kitahara for their technical assistance.

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Accepted April 14, 2004