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Immunohistochemical study of the upper surface layer in rat mandibular condylar cartilage

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Summary. Both hyaluronic acid and fibronectin localizations were examined in the upper surface layer of rat mandibular condylar cartilages by immunohistochemical techniques. Their delicate structure was successfully preserved by preparation procedures of joint condyles with disks. Paraformaldehyde-fixed cartilaginous tissues were cut in a cryostat, and cryosections were analyzed using streptavidin-peroxidase and indirect immunofluorescence methods. Another immunogold method with conventional preparation procedures and a quick-freezing method was performed for their ultrastructural analyses. Both hyaluronic acid-binding protein and anti-fibronectin antibody were used to localize hyaluronic acid and fibronectin in the mandibular condylar cartilage, respectively. Some cryosections were pre-treated with hyaluronidase and chondroitinase before such labeling. The upper surface layer was composed of double laminar structures. One bordered with the cartilage matriceal surface, which was positive for fibronectin. The hyaluronic acid was localized over the fibronectin layer. Therefore, the hyaluronic acid in vivo was bound with fibronectin in the cartilaginous matrix, performing lubrication for the mandibular joint movement.

Key words: Articular cartilage, Fibronectin, Hyaluronic acid, Temporomandibular joint, Upper surface layer

Introduction

The temporomandibular joint (TMJ) is one of the most complex structures in the maxillofacial regions, and involves two separate synovial joints, which usually function harmoniously at each joint movement (Hawthorn and Flatau, 1990). Moreover, the articular surface tissues of both disk and condyle are usually in close relation during hinge and gliding movement of the TMJ, always leaving the articular cartilage surface exposed to frictional and loading forces (Axelsson et al., 1992; Iwai-Liao et al., 1994; Roth et al., 1997).

Concerning superficial layer components of the general articular cartilage in animals, most of the studies have been done for long bones of hip or knee joints. The localization of fibronectin on the most superficial layer, the so-called "lamina splendens" (MacConail, 1951), has already been reported for the rabbit femoral articular cartilage (Nishida et al., 1995). In addition, the presence of hyaluronic acid was demonstrated on the cartilage surface of human hip femoral heads (Asari et al., 1994). In the past decade, we have already clarified ultrastructures of the upper surface layers in articular cartilages of rat femoral heads and condyles by our “in vivo cryotechnique” followed by the freeze-substitution method for electron microscopy, and also compared them with those revealed by the conventional preparation methods with chemical fixatives (Toriumi et al., 1996; Leng et al., 1998; Watanabe et al., 2000). In those reports, our close examination of the hip or knee articular cartilages demonstrated fairly good preservation of native morphology at the frozen upper surface cartilage. The upper surface layer in situ was found to be thicker than that in the conventionally prepared specimens, in which many granular structures with different sizes and varied electron-density were localized together with filamentous networks, indicating that it contained some amounts of lipid components (Watanabe et al., 2000). Concerning the mandibular condylar cartilage, we have demonstrated non-collagenous microfibrilar networks, which were associated with lipid-like structures, in the upper articular surface tissues after successfully preserving the delicate morphology with the quick-freezing and deep-etching method (manuscript in preparation). Although the presence of lipids and hyaluronic acid on the articular surfaces has been reported in other animal joints (Hills, 1989; Mills et al., 1994; Ayman and Mohamed, 1996; Toriumi et al., 1996; Leng et al., 1998; Watanabe et al., 2000; Rahamin et al., 2001), there is

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still a lack of consensus concerning the localization and role of hyaluronic acid and fibronectin in the upper surface cartilage layer of the TMJ. In the present study, we have used immunohistochemical techniques for light microscopy and confocal laser scanning microscopy, and another post-embedding immunogold method for electron microscopy. We focused especially on hyaluronic acid and fibronectin localizations in the upper surface layer of rat mandibular condylar cartilages in order to get a better understanding about in vivo functions of the upper surface layer. Additionally, the immunolocalizations of those molecules in the articular disk and synovial tissues of the TMJ are presented in order to clarify their origins.

Materials and methods

Twenty-five Wistar rats, weighing approximately 200-250g, were deeply anesthetized with sodium pentobarbital, and perfused with 2% paraformaldehyde in 0.1M phosphate buffer (PB), pH 7.4, via hearts. While TMJ were dissected, our special attention was given to preserving the in situ condyles with attached articular disks in order to leave the inner articular cartilage structure intact. The removed condylar specimens were additionally fixed with 2% paraformaldehyde in 0.1M PB at 4 °C for 1 hour. After being rinsed in phosphate-buffered saline (PBS), they were immersed in 5% EDTA, pH 7.2, at 4 °C for 5 days. Subsequently, they were immersed with 20% sucrose and 10% glycerol in 0.1M PB at 4 °C overnight, embedded in OCT compound, frozen at -80 °C and sagittaly sectioned at 9 μm thickness in a cryostat (Microm, Walldorf, Germany). The present study was performed in accordance with the guidelines governing animal experiments within the University of Yamanashi.

An anti-fibronectin antibody obtained from rabbits is commercially available (Sigma Chemical, St. Louis, MO, USA). A biotinylated hyaluronic acid binding protein (B-HABP) was also commercially obtained (Seikagaku Co., Tokyo, Japan). Some serial sections were pre-treated with protease-free chondroitinase ABC at a concentration of 1 U/100 μl (Seikagaku Co., Tokyo, Japan) in Tris-HCl buffer, pH 8.0, at 37 °C for 30 min and others were pre-treated with hyaluronidase derived from Streptomyces hyalurolyticus (Amano Enzyme Inc., Nagoya, Japan) at a concentration of 192 TRU (Turbidity Reducing Unit) in citric acid-acetate buffer, pH 6.0, at 37 °C for 1 hr.

Peroxidase labeling for light microscopic analyses

Some cryosections were first treated with 0.7% hydrogen peroxide in phosphate-buffered saline (PBS) for 15 min to inhibit the enzyme reaction of endogenous peroxidase. After being washed with PBS, they were blocked with 10% BSA for 30 min, and then incubated with rabbit anti-fibronectin antibody at a dilution of 1:100 with 1% BSA in PBS at room temperature for 1 hr. They were then rinsed in PBS three times for 5 min each. Subsequently, they were incubated with secondary anti-rabbit IgG antibody labeled with peroxidase (Histofine Simple Stain Kit; Nichirei, Tokyo, Japan) for 30 min and visualized for peroxidase enzyme reactions with a metal-enhanced 3,3'diaminobenzidine (DAB) kit (Pierce, Rockford, IL, USA) and 0.02% stable hydrogen peroxide buffer for 5 min. For immunocontrol staining, the first incubation step with the primary antibody was omitted.

To examine hyaluronic acid on the articular surface cartilage, some cryosections were incubated with biotinylated hyaluronic acid-binding protein (B-HABP) at a dilution of 1:150 in PBS containing 1% BSA at room temperature for 1 hr, subsequently incubated with the streptavidin-peroxidase solution (Histofine SAB-PO Kit; Nichirei, Tokyo, Japan) for 5 min, and treated with the metal-enhanced DAB kit (Pierce, Rockford, IL, USA) for 5 min. For specific B-HABP labeling control, other cryosections were incubated with PBS instead of B-HABP.

Confocal laser scanning microscopy

For fluorescence labeling, some cryosections were routinely blocked with 10% BSA for 10 min and then incubated with the rabbit anti-fibronectin antibody at a dilution of 1:100 in PBS containing 1% BSA at room temperature for 1 hr. Subsequently, they were incubated with secondary anti-rabbit IgG antibody labeled with Alexa (594 nm) (Molecular Probes, Eugene, OR, USA) for 30 min, and mounted on slide glasses with the vectashield (Vector Lab., Burlingame, CA, USA). To examine hyaluronic acid localization on the articular cartilage, other cryosections were incubated with B-HABP at a dilution of 1:100 in PBS containing 1% BSA at room temperature for 1 hr, then with streptavidin coupled to Alexa (488 nm) for 30 min and mounted on slide glasses with the vectashield.

For double-fluorescence labeling for fibronectin and hyaluronic acid, cryosections were incubated with a mixture of the primary anti-fibronectin antibody and B-HABP at dilutions of 1:100 at room temperature for 1 hr, and subsequently incubated with secondary anti-rabbit IgG antibody coupled to Texas red and another streptavidin coupled to Alexa (488 nm) mixed in equal amounts at dilutions of 1:200 for 30 min.

Confocal laser scanning microscopy for a non-decalcified specimen

Five Wistar rats were deeply anesthetized with sodium pentobarbital, and perfused with 2% paraformaldehyde via hearts. Their temporomandibular joints were removed, as described above, immersed in 20% sucrose and 10% glycerol in 0.1M PB at 4 °C overnight, embedded in OCT compound and frozen at -80 °C. They were sagittaly sectioned at 9 μm thickness in a cryostat (Microm, Walldorf, Germany) without

decalcification. The prepared sections were then treated for immunohistochemistry, as described in the previous section.

Post-embedding immunoelectron microscopy

Dissected TMJ condyles with disks were postfixed with 2% PF and 1% glutaraldehyde in 0.1M PB at 4 ˚C for 1 hr. After being washed in 0.1 M PB for 1 hr, they were decalcified in 8% EDTA, pH 7.4, at 4˚C for 2 weeks. Then, the specimens were cut into two pieces with a razor blade, leaving each half of the condyle with its respective half of the disk. Subsequently, they were dehydrated in a graded series of ethanol, embedded in LR gold at -30 °C, placed in small plastic capsules and polymerized under ultraviolet light (UV) at -30 °C for 24 hrs. Some ultrathin sections were cut at 70 nm thickness on an ultramicrotome and then mounted on Formvar-coated nickel grids. They were incubated with the B-HABP at a dilution of 1:150 at room temperature for 1 hr. After being washed in PBS, they were additionally incubated with goat anti-biotin antibody conjugated with gold particles of 10nm diameter (British Biocell International, UK) in a moist chamber at room temperature for 1 hr. Then they were washed in PBS five times and additionally incubated with rabbit anti-fibronectin IgG antibody (Sigma Chemical, St. Louis, MO, USA) on the same side of sections at a dilution of 1:100 at room temperature for 1 hr. After being washed in PBS, they were incubated with goat anti-rabbit IgG antibody conjugated with gold particles of 15 nm in diameter. They were finally counterstained with uranyl acetate for 3 min and examined by a Hitachi H-7500 transmission electron microscope.

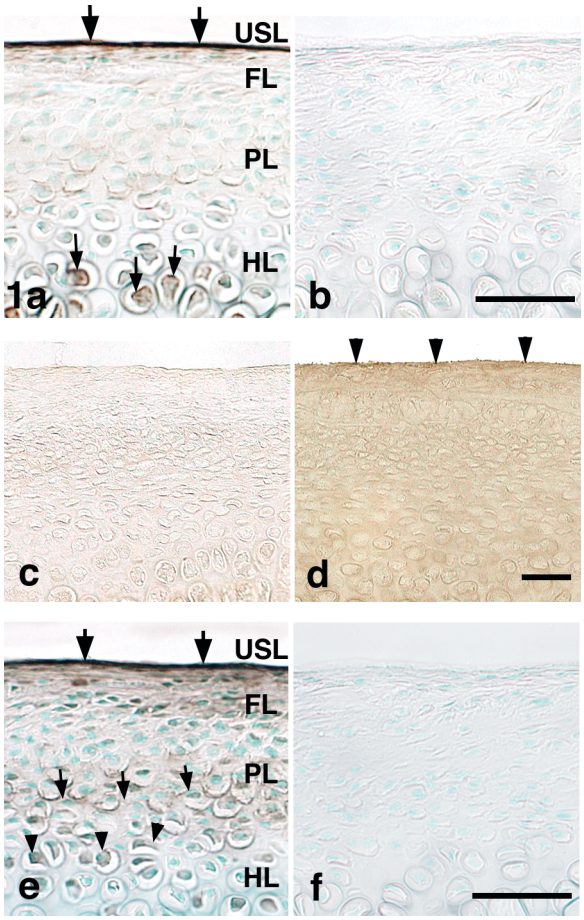

Fig. 1. Light microscopic localization of hyaluronic acid and fibronectin in rat mandibular condylar cartilages. **a.** Some DAB reaction for HA is clearly localized along the upper surface layer (USL, large arrows). Another reaction for HA is slightly observed in the hypertrophic layer (HL, small arrows). **b.** Control specimens without B-HABP. **c.** After pre-treatment with hyaluronidase, DAB reaction products for hyaluronic acid are completely abolished in all layers. **d.** After pre-treatment with chondroitinase, the DAB reaction for hyaluronic acid is enhanced in the extracellular matrix, whereas that in the upper surface layer is changed and appears as irregular and dotted patterns (arrowheads). **e.** Immunohistochemical micrograph obtained from serial sections of the condylar cartilages demonstrating fibronectin immunoreaction products. Intense DAB reaction products are detected in the upper surface layer (USL, large arrows). Another positive immunoreaction is seen in the extracellular matrix of the proliferative layer (PL, small arrows). They are slightly localized in some chondrocytes of the hypertrophic layer (HL, arrowheads). **f.** Immunocontrol specimens without the anti-fibronectin antibody. USL: upper surface layer, FL: fibrous layer, PL: proliferative layer, HL: hypertrophic layer. Scale bar: 50 μm.

Quick-freezing and freeze-substitution method

Three Wistar rats were anesthetized with sodium pentobarbital and their temporomandibular joints were carefully dissected. Subsequently, articular disks were slightly cut open with razor blades, and articular cartilages were exposed. They were quickly frozen with liquid isopentane-propane cryogen (-193 °C) cooled with liquid nitrogen, as reported before (Toriumi et al., 1996; Leng et al., 1998; Watanabe et al., 2000). The frozen specimens were transferred into liquid nitrogen and their well-frozen parts were trimmed off and fractured into small pieces with a cooled surgical blade in the liquid nitrogen.

The specimens were then processed for the freeze-substitution method, as reported before (Leng et al., 1998; Watanabe et al., 2000). They were transferred into absolute acetone containing 2% paraformaldehyde at -80 ˚C and kept for 24 hrs. Then, they were put into a deep-freezer at -25 ˚C for 2 hrs, embedded in LR-Gold, sectioned and processed for the post-embedding immunohistochemistry, as described in the previous section.

Results

By the streptavidin-peroxidase technique, DAB reaction products for hyaluronic acid were mainly localized along the articular cartilage surface areas (Fig. 1a). In the deep cartilaginous matrix, they were also localized in the hypertrophic chondrocyte layer, especially in pericellular regions of chondrocytes. On the

contrary, the control B-HABP section showed no DAB reaction products (Fig. 1b). Moreover, in some specimens pre-treated with the hyaluronidase before B-HABP labeling, such DAB reaction products for hyaluronic acid were completely abolished in all cartilage layers (Fig. 1c). In other specimens pre-treated with chondroitinase, DAB reaction products for hyaluronic acid were considerably enhanced in the extracellular matrix, but they were decreased in the upper cartilage surface (Fig. 1d).

In some cryosections immunostained with anti-fibronectin antibody, a linear immunoreaction pattern was detected along the cartilage surface area (Fig. 1e), as compared with that in immunocontrol cryosections (Fig. 1f). The intensity of immunoreaction products for fibronectin decreased in the fibrous layer, but increased in the matrices of the proliferative layer. They were also localized in some chondrocytes in the hypertrophic layer.

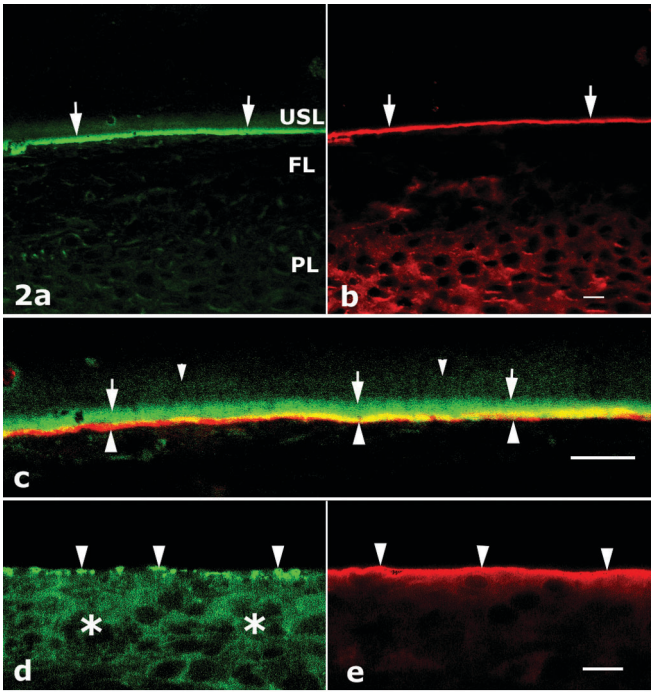

Fig. 2. Confocal laser scanning micrograph for hyaluronic acid and fibronectin localizations in rat mandibular condylar cartilages. **a.** The fluorescence reaction for hyaluronic acid is clearly observed in the upper surface layer (USL, large arrows). The fluorescence labeling pattern is thick and similar to that revealed by the immunoperoxidase technique. **b.** Another fibronectin immunoreaction is also observed as a continuous linear pattern along the upper surface layer (arrows). **c.** Double fluorescence overlapping images show the intense labeling of hyaluronic acid (arrows) and fibronectin (large arrowheads). The upper surface layer is labeled as a bi-layer pattern in some parts. In addition, the hyaluronic acid fluorescence reaction is slightly observed in some remnants of synovial fluid (small arrowheads). **d.** Double fluorescence labeling micrograph for hyaluronic acid and fibronectin obtained from the same section pre-treated with chondroitinase. The positive fluorescence reaction for hyaluronic acid is considerably decreased, mainly in the upper surface layer (arrowheads), but the extracellular matrix enhanced the fluorescence reactivity (star marks). **e.** The positive immunoreaction for fibronectin exhibits no apparent change in the identical upper surface layer (arrowheads). USL: upper surface layer, FL: fibrous layer, PL: proliferative layer. Scale bar: 10 μm.

By indirect fluorescence labeling for hyaluronic acid, a positive reaction was detected along the upper surface layer with a thick linear pattern (Fig. 2a). This positive fluorescence reaction was also detected in the pericellular region of hypertrophic chondrocytes. In addition, another fibronectin immunoreaction was detected as a continuous linear pattern along the cartilage surface (Fig. 2b). In the extracellular matrix, immunoreaction products for the fibronectin were localized in the proliferative layer. When the double overlapping fluorescence image was taken, the fibronectin fluorescence remained close to the cartilaginous matrix as a linear pattern (Fig. 2c). However, the hyaluronic acid had thicker fluorescence reaction products with brighter intensity and overlapped each other in some parts with the fibronectin to form a bi-layer structure (Fig 2c). In some cryosections pre-treated with chondroitinase, fluorescence reaction products for the hyaluronic acid were partially abolished along the cartilage surface areas, but the extracellular matrix exhibited them at a remarkably increased level (Fig. 2d). However, immunostaining for fibronectin showed almost the same unchanged pattern (Fig. 2e), as described for the streptavidin-peroxidase immuno-reaction.

On non-decalcified sections, positive fluorescence reaction products for both hyaluronic acid and fibronectin were observed along the inferior and superior surface parts of the articular disk at low magnification (Fig. 3a-c). Moreover, some remnants of synovial fluid positive for hyaluronic acid were observed as a weakly stained band along the inferior surface of the disk (Fig. 3 d-f).

On serial sections of the similar non-decalcified specimens (Fig. 4a-c), strong positive fluorescence reaction products for hyaluronic acid were observed in the synovial tissue of the TMJ. The synovial fluid was weakly stained on the synovial tissue surfaces facing the articular cavity. Positive immunoreaction products for fibronectin were also seen to delineate the surface areas of the articular cartilage and the synovial tissues.

The double-labeling for both hyaluronic acid and fibronectin in the upper surface layer was performed by the post-embedding immunogold method at electron microscopic level (Fig. 5). Many small immunogold particles with 10 nm diameter for hyaluronic acid were localized along the electron-dense surface layer, coexisting with immunolgold particles of 15 nm in diameter for fibronectin (Fig. 5a, inset). Some remnants of synovial fluid, resembling “projections” extending from the cartilage surface, appeared to be labeled only with immunogold particles of 10 nm diameter, indicating the presence of hyaluronic acid (Fig. 5b, inset).

By the quick-freezing and freeze-substitution methods, a well-preserved upper surface layer was observed (Fig. 6). With a thicker appearance of the

surface layer than that prepared by the conventional preparation method, it appeared to be clearly labeled with immunogold particles of 10 nm and 15 nm in size for hyaluronic acid and fibronectin, respectively. Moreover, the upper surface layer appeared to be composed of microfibril-like structures (Fig. 6, inset). Some synovial fluid "projections" were also observed to be labeled with immunogold particles of 10 nm in size, indicating hyaluronic acid localization.

Discussion

In the present study, both hyaluronic acid and fibronectin were detected in the upper surface layer of the rat mandibular condylar cartilage, articular disk surface areas and synovial tissues. Although the conventional chemical fixation data for light and confocal laser scanning microscopic analyses has mainly been presented, we obtained good preservation of the cartilage surface structure because the articular cartilage specimens were carefully treated for dissection of the condyle including articular capsules and disks in order to avoid damaging the articular cartilage surface. By such protection of the cartilage surface during the preparation processes, we detected some remnants of synovial fluid in almost all the decalcified and non-decalcified specimens, especially in those observed by confocal laser scanning microscopy and electron microscopy. These synovial fluid remnants observed in the condylar cartilage and disk surfaces were positive for the hyaluronic acid-binding protein. Moreover, the synovial tissue exhibited strong positive reaction products for hyaluronic acid, so we could postulate a possibility that hyaluronic acid detected in the articular surfaces originated from the synovial tissue. On the contrary, positive immunoreaction products for fibronectin were not detected in the synovial fluid remnants, but it was clearly demonstrated that fibronectin delineated the matriceal surfaces of the condylar cartilage, continued with the synovial tissue surface and then reversed to the lower surface of the articular disk. These findings allow

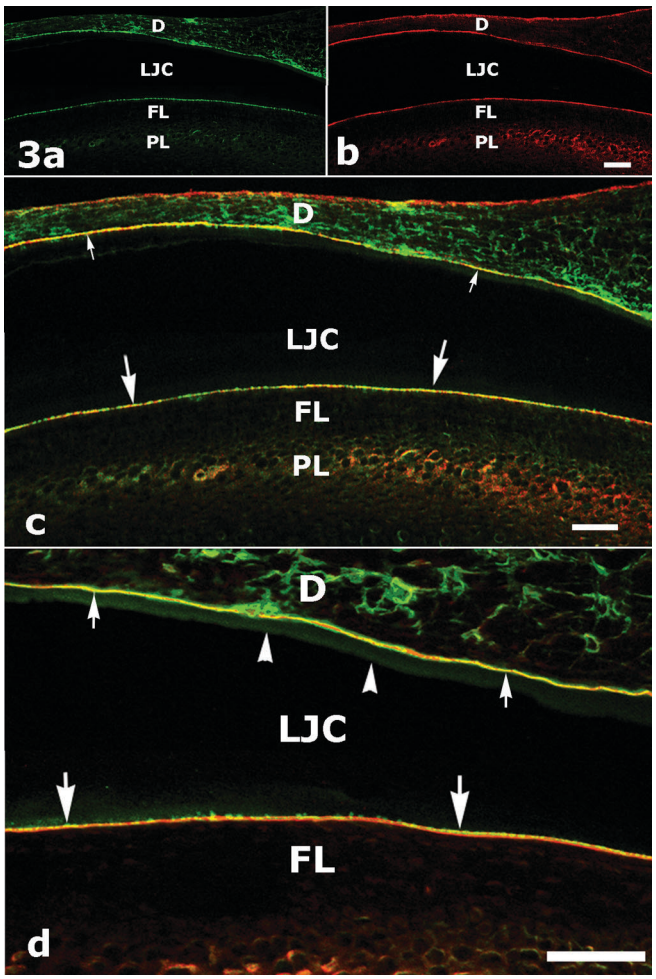

Fig. 3. Confocal laser scanning micrographs of the TMJ, showing a positive fluorescence reaction for both hyaluronic acid **(a)** and fibronectin **(b)** in the upper surface layer of the condylar cartilage (**c**, large arrows) and at the lower surface of the articular disk (**c**, small arrows). **d.** Higher magnification of the TMJ showing synovial fluid remnants weakly stained for hyaluronic acid in the lower surface of the disk (arrowheads). The double fluorescence labeling shows some overlapped patterns for both fibronectin and hyaluronic acid in the condylar upper surface layer (large arrows) and the inferior surface of articular disks (small arrows). D: disk, LJC: lower joint cavity, FL: fibrous layer, PL: proliferative layer. Scale bar: 50 μm.

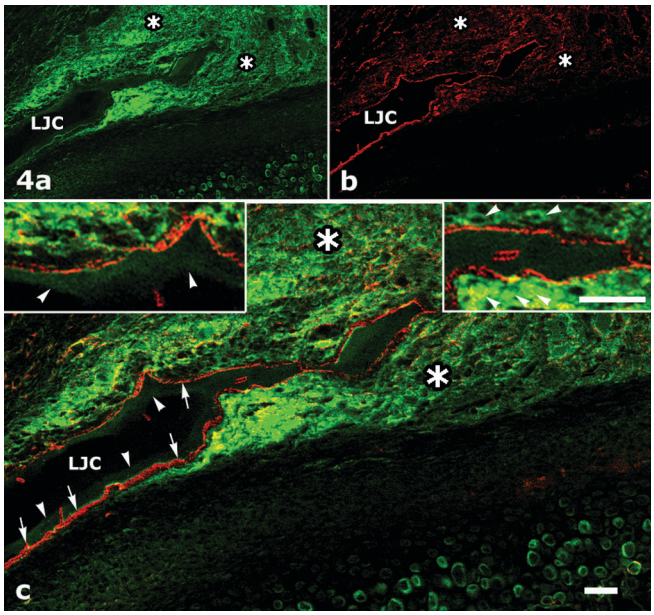

Fig. 4. Confocal laser scanning micrographs of the synovial tissues (asterisks), demonstrating positive fluorescence reaction products for hyaluronic acid **(a)** in synoviocytes (c, upper right inset, arrowheads) and also synovial fluid weakly stained for hyaluronic acid on the surface areas of synovium (**c**, upper left inset, arrowheads). Another immunoreaction for fibronectin **(b)** is observed to delineate the surface areas of synovial tissues and the articular cartilage (**c**, arrows). LJC: lower joint cavity. Scale bar: 50 μm.

us to consider that fibronectin might be a product from the fibrous layer in the condylar cartilage and also from the extracellular matrix in the disk and synovial tissues. Concerning the two enzyme treatments, the specimens pre-treated with hyaluronidase exhibited no DAB reaction products for the hyaluronic acid-binding protein. Other specimens pre-treated with chondroitinase exhibited enhanced DAB reaction products for hyaluronic acid in the extracellular matrix, but they decreased in the upper surface layer. This is reasonable because chondroitinase hydrolyses hyaluronic acid at a slower rate than chondroitin sulphate (Suzuki et al., 1968; Inkinen et al., 1999).

It has been suggested that the upper surface layer of the articular cartilage was usually changed during routine tissue dissection, double-chemical fixation and

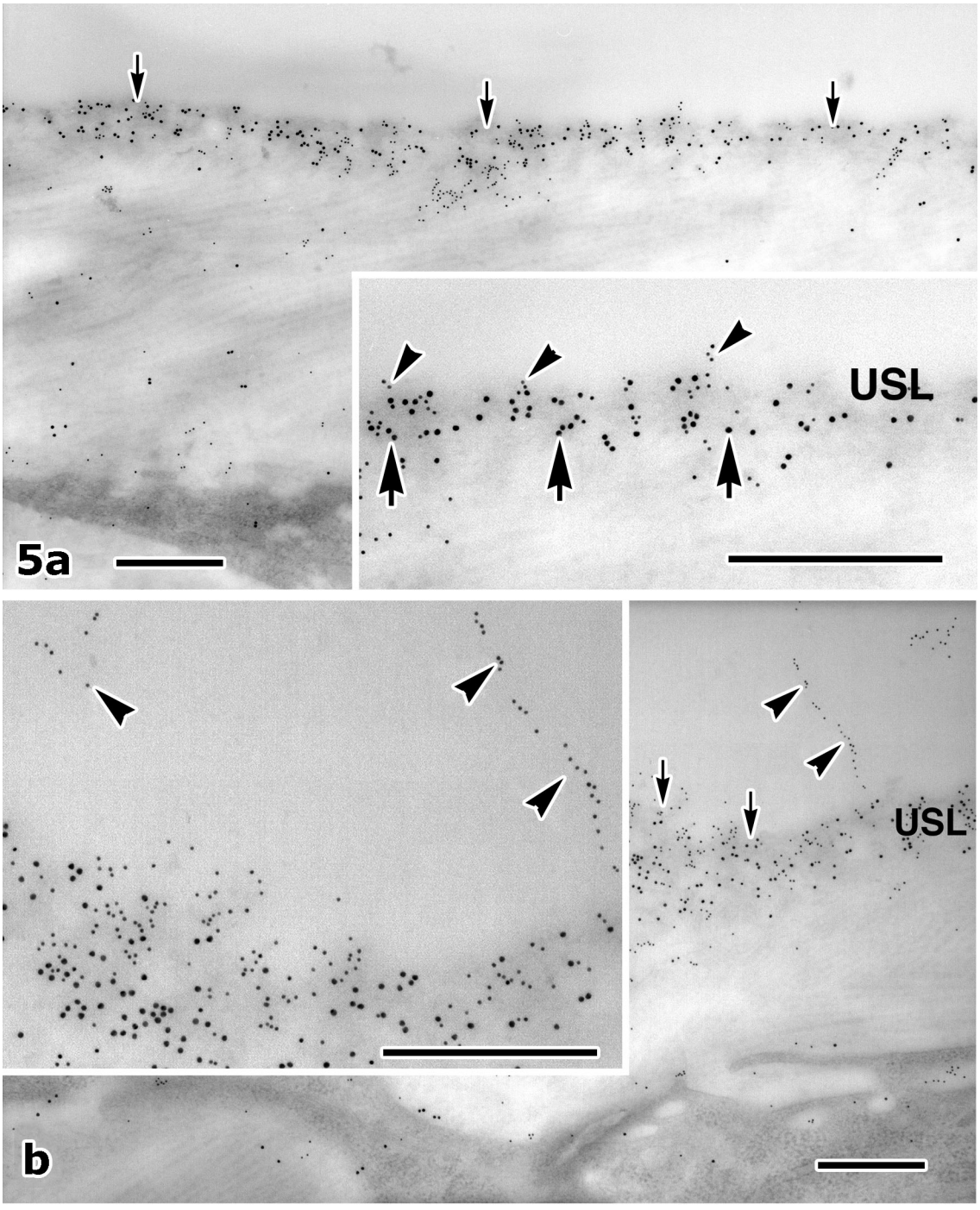

Fig. 5.a. Immunoelectron micrographs of the upper surface layer labeled for fibronectin and hyaluronic acid (small arrows). The double labeling shows gold particles of 10 nm in diameter for hyaluronic acid (arrowheads) and other gold particles of 15 nm in diameter for fibronectin (large arrows) in the electron-dense upper surface layer. Inset: Higher magnification. USL: Upper surface layer. Scale bar: 0.5 μm. **b.** Some remnants of synovial fluid (arrowheads) are observed to be labeled with gold particles of 10 nm in diameter, corresponding to hyaluronic acid localization. Arrows indicate the upper surface layer, positive for both hyaluronic acid and fibronectin. Inset: Higher magnification. Scale bar: 0.5 μm. USL: upper surface layer.

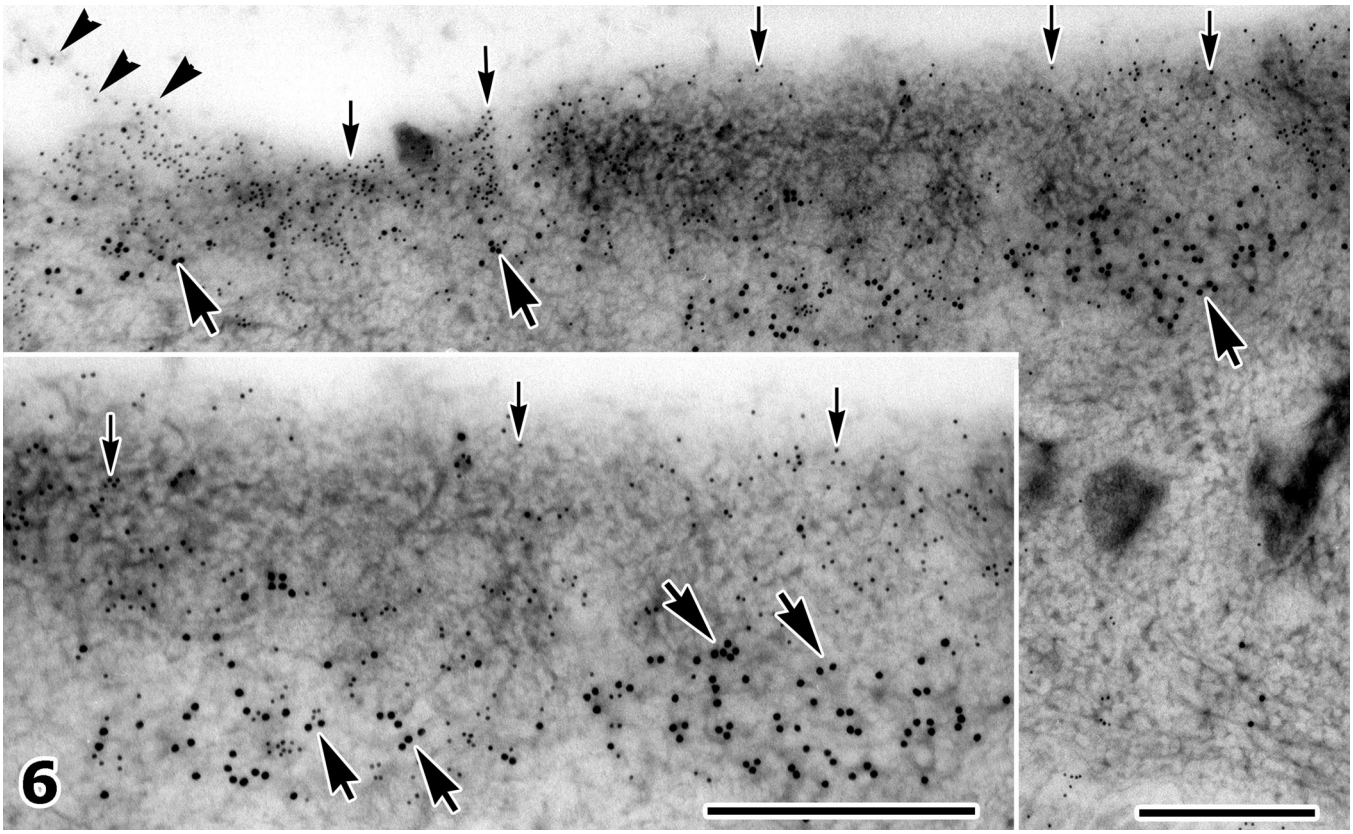

Fig. 6. Immunoelectron micrographs of the upper surface layer of the condylar cartilage prepared by the quick-freezing and freeze-substitution methods. The surface areas appear to be thick and composed of microfibril-like structures. Positive immunogold labeling for HA is observed along the cartilage surface areas (small arrows) and at the synovial fluid remnants (arrowheads). Colocalization with immunogold particles of 15 nm in size, corresponding to fibronectin (large arrows), is also observed in the upper surface layer. Inset: Higher magnification. Scale bar: 0.5 μm.

routine buffer washing. Therefore, in our previous studies, we used several different preparation techniques including conventional chemical fixation and the quick-freezing and freeze-substitution methods in order to examine native morphological features (Watanabe et al., 2000). In the present study, by the quick-freezing and freeze-substitution methods, we were able to demonstrate a more natural appearance of the articular cartilage surface. The upper surface layer was seen to be more continuous and thicker than that prepared by conventional chemical fixation. Moreover, immunolabeling for both hyaluronic acid and fibronectin appeared to be more compactly localized along the upper surface layer.

A functional role of hyaluronic acid as a main component for lubrication has been controversial with evidence supporting that the surface-active phospholipids are responsible for boundary lubrication (Hills, 1989; Dijkgraaf et al., 1995; Toriumi et al., 1996; Watanabe et al., 2000; Rahamin et al., 2001), but the present study has clearly demonstrated that hyaluronic acid was compactly present in the upper surface layer of the condylar cartilage and lower surface areas of the articular disk, and also bound with fibronectin, as suggested before (Yamada et al., 1980; Clemmensen et al., 1982; Nishida et al., 1995). Moreover, these two components probably coexist with some phospholipids to form a functional barrier structure at the articular surfaces for cartilage protection as well as for the lubricant structure.

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References

Asari A., Miyauchi S., Machida A., Kohno K. and Uchiyama Y. (1994). Localization of hyaluronic acid in human articular cartilage. J. Histochem. Cytochem. 42, 513-522.

Axelsson S., Holmlund A. and Hjerpe A. (1992). Glycosaminoglycans in normal and osteoarthrotic human temporomandibular joint disks. Acta Odontol. Scan. 50, 113-119.

Ayman M. and Mohamed S. (1996). Histochemical and inmunohistochemical studies of the effects of experimental anterior

disc displacement on sulfated glycosaminoglycans, hyaluronic acid, and link protein of the rabbit craniomandibular joint. J. Oral Maxillofac. Surg. 54, 992-1003.

Clemmensen I., Holund B., Johansen N. and Andersen R.B. (1982). Demonstration of fibronectin in human articular cartilage by indirect immunoperoxidase technique. Histochemistry 76, 51-56.

Dijkgraaf L., Lambert G., Boering G. and Liem R. (1995). Normal cartilage structure, biochemistry and metabolism: A review of the literature. J. Oral Maxillofac. Surg. 53, 924-929.

Hawthorn R. and Flatau A. (1990). A textbook and colour atlas of the temporomandibular joint. Norman J.E. de B. and Bramley P. (eds). Wolf Medical Publications. Ipswich. pp 1-25.

Hills B.A. (1989). Oligolamellar lubrication of joints by surface active phospholipid. J. Rheumatol. 16, 82-91.

Inkinen R.I., Lammi M.J., Agren R., Tammi U., Puustjarvi K. and Tammi M.I. (1999). Hyaluronan distribution in the human and canine intervertebral disc and cartilage endplate. Histochem. J. 31, 579-587.

Iwai-Liao., Ogita Y., Tsubai T. and Higashi Y. (1994). Scanning electron microscopy of elastic fibers in the articular disc of the rat mandibular joint. Okajimas Folia Anat. Jpn. 71, 211-226.

Leng C-G., Yu Y., Ueda H., Terada N., Fujii Y. and Ohno S. (1998). The ultrastructure of anionic sites in rat articular cartilage as revealed by different preparation methods and polyethyleneimine staining. Histochem. J. 30, 253-261.

MacConail M.A. (1951). The movements of bones and joints. IV. The mechanical structure of articular cartilage. J. Bone Joint Surg. Br 33, 251-257.

Mills K., Fiandaca D. and Scapino R. (1994). Morphologic, microscopic and immunohistochemical investigations into the function of the primate TMJ disc. J. Orofac. Pain 8, 136-154.

Nishida K., Inoue H. and Murakami K. (1995). Immunohistochemical demonstration of fibronectin in the most superficial layer of normal rabbit articular cartilage. Ann. Rheum. Dis. 54, 995-998.

Rahamin E., Better H., Dagan A. and Nitzan D.W. (2001). Electron microscope and biochemical observations of the surface-active phospholipids on the articular surfaces and in the synovial fluid of the temporomandibular joint: A preliminary investigation. J. Oral Maxillofac. Surg. 59, 1326-1332.

Roth S., Muller K., Fischer D. and Danhauer K. (1997). Specific properties of the extracelular chondroitin sulphate proteoglycans in the mandibular condylar growth center in pigs. Archs. Oral Biol. 42, 63-76.

Suzuki S., Saito H., Yamagata T., Anno K., Seno N., Kawai T., and Furuhashi T. (1968). Formation of three types of disulfated disaccarides from chondroitin sulfates by chondroitinase digestion. J. Biol. Chem. 243, 1543-1550.

Toriumi H., Nakagawa H., Ueda H., Leng C-G., Fujii Y. and Ohno S. (1996). Proteoglycans in articular cartilage revealed with a quick-freezing and deep-etching method. Ann. Rheum. Dis. 55, 466-474.

Watanabe M., Leng C-G., Toriumi H., Hamada Y., Akamatsu N., and Ohno S. (2000). Ultrastructural study of upper surface layer in rat articular cartilage by “in vivo cryotechnique” combined with various treatments. Med. Electron Microsc. 33, 16-24.

Yamada K., Kennedy D., Kimata K. and Pratt R. (1980). Characterization of fibronectin interactions with glycosaminoglycans and identification of active proteolytic fragments. J. Biol. Chem. 255, 6055-6063.

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