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Immunohistochemical localization of phosphatidylcholine in rat mandibular condylar surface and lower joint cavity by cryotechniques

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Summary. The immunolocalization of phospholipids has not yet been clearly demonstrated in temporomandibular joints (TMJs). We have examined the distribution of one of phospholipids, phosphatidylcholine (PC), in the rat mandibular condylar surface and lower joint cavity. Some fresh resected TMJs with their disks attached were immediately plunged into isopentane-propane cryogen (-193 °C). Cryostat sections were cut, mounted on NH_3^+-coated slides, and fixed with paraformaldehyde (PF). Cryosections were first immunostained with anti-mouse PC antibody (JE-1). Subsequently, they were labeled with immunogold particles following silver enhancing for light microscopic analyses. Some cryosections were subjected to double immunofluoresecence labeling with anti-fibronectin antibody or hyaluronic acid-binding protein in combination with the anti-PC antibody. As an immunocontrol, other cryosections were pretreated with phospholipase A_2 before such immunofluorescence labeling. We have confirmed the presence of PC in the lower joint cavity of rat TMJs as well as on the mandibular condylar surface layer, which was colocalized with hyaluronic acid and fibronectin respectively. However, by treatment with phospholipase A_2, such immunolabeling for PC was clearly decreased, showing that the PC is a component in the rat in vivo TMJ. These findings suggest that PC, hyaluronic acid and fibronectin may interact each other in the TMJ articular surface areas to play a functional role for lubrication in TMJ.

Key words: Mandibular condylar cartilage, Lower joint cavity, Upper surface layer, Phosphatidylcholine, Joint lubrication

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Introduction

It was reported that hyaluronic acid was a functional component as the predominant articular boundary lubricant of hip or knee joints (Ogston and Stanier, 1953), but another role of the hyaluronic acid within frameworks of the joint's lubrication system has been questioned until now (Nitzan et al., 2001). In addition, several studies reported that surface-active phospholipids (SAPL) acted as major and highly efficient boundary lubricants, protecting hip or knee articular surfaces (Hills and Butler, 1984; Hills, 1990; Schwarz and Hills, 1998; Watanabe et al., 2000; Sarma et al., 2001). Some biochemical analyses of the articular surface components and synovial fluid have already revealed the presence of phosphatidylcholine (PC) (Hills and Butler, 1984; Sarma et al., 2001). It was also reported that traces of phosphatidylethanolamine and sphingomyelin were found on the surface areas of articular cartilage tissues (Hills and Butler, 1984; Sarma et al., 2001). A glycoprotein termed as "lubricin" was isolated from the load-bearing fraction of the synovial fluid and identified as a major boundary lubricant (Radin et al., 1971; Swann et al., 1985). In addition, the lubricin was demonstrated to function as a carrier protein for the highly insoluble SAPL, which deposited on the articular surface tissue as an oligolamellar layer (Schwarz and Hills, 1998).

An electron microscopic study of articular cartilage surfaces of sheep knee joints treated with tannic acid and glutaraldehyde fixation, demonstrated the presence of multilamellar structures of 200 nm to 300 nm in diameter on the articular surfaces (Hills, 1990). The lamellar bodies present in type B synoviocytes were suggested as a primary source of the SAPL (Schwarz and Hills, 1998). In the human temporomandibular joint (TMJ), electron microscopic findings suggested the presence of phospholipids on the surface of articular cartilages (Rahamim et al., 2001). The amorphous and highly osmophilic material on the articular surface was

shown to include membrane-bound vesicles with lamellated pattern surrounding the amorphous-dense core. Biochemical extraction revealed PC as the major component of the polar lipids. We have already reported another presence of lipid-like structures in rat femoral or knee articular cartilage surfaces by electron microscopic studies in combination with the quick-freezing and freeze-substitution method (Watanabe et al., 2000). In addition, we have applied the quick-freezing method for examining the upper surface layer in situ of the rat TMJ cartilage (Yoshida et al., 2004). Recently, we demonstrated that hyaluronic acid (HA) and fibronectin (FN) were localized as a double layer on the articular surfaces of mandibular disks and condyles (Zea-Aragon et al., 2004a), and suggested that both HA and FN might have a principal role in boundary lubrication, interacting with the SAPL. However, there has been no immunohistochemical report, by using specific antibody against PC, about the distribution of SAPL in the TMJ. The purpose of the present study is to examine the immunolocalization of phosphatidylcholine (PC) with other components, such as HA or FN, in rat TMJs to get a better understanding of its role in the lubrication system.

Materials and methods

Antibodies and specific probe

An anti-phosphatidylcholine antibody (JE-1, Nam et al., 1990) was a gift from Dr. M. Umeda (Rinshoken, Tokyo, Japan). For double immunohistochemical labeling, a rabbit polyclonal anti-fibronectin antibody was commercially available (Sigma Chemical, St. Louis, MO, USA). A biotinylated hyaluronic acid binding protein (B-HABP) was also commercially obtained from Seikagaku Co., Tokyo, Japan. As such immunolabeling and probe-labeling controls, neither primary antibodies nor B-HABP were used in the similar way (data not shown), as described before (Zea-Aragon et al., 2004a,b).

Quick-freezing method and immunogold labeling for light microscopy

The present study was performed in accordance with the guidelines governing animal experiments within University of Yamanashi. Five Wistar rats, weighing approximately 200-250 g, were deeply anesthetized with sodium pentobarbital. Their TMJs were dissected with their disks attached and immediately plunged into liquid isopentane-propane cryogen (-193 °C) to keep TMJs intact. Subsequently, they were transferred into liquid nitrogen and embedded in OCT compound. They were sagittaly cut at 9 µm thickness in a cryostat apparatus (Microm, Walldorf, Germany).

The cryosections mounted on Matsunami Adhesive Slides (MAS; highly densed NH_3^+-coated slides (Matsunami Glass, Osaka, Japan)) were fixed in 2% paraformaldehyde (PF) at 4 °C for 15 min. They were blocked with 1% BSA in PBS at room temperature for 30 min. Subsequently they were incubated with a primary anti-phosphatidylcholine antibody, JE-1 (1:100 at dilution), at room temperature for 1 hr. After being washed in PBS, they were incubated with secondary goat anti-mouse IgM antibody with gold particles of 10 nm in diameter (British Biocell International, UK) at room temperature for 1 hr. Then they were washed in PBS and incubated with a freshly mixed silver-enhancing solution at room temperature for a period of 5 min. After being washed in distilled water to stop the silver-enhancing reaction, they were mounted on slide glasses with glycerol.

Double immunofluorescence labeling for phosphatidylcholine (PC) and hyaluronic acid (HA)

Some serial cryosections obtained from the prepared specimen, as described in the previous section, were incubated with the anti-PC antibody at a dilution of 1:100 in PBS containing 1% BSA at room temperature for 1 hr. Subsequently, they were incubated with the secondary anti-mouse IgM antibody labeled with FITC (594 nm) (Molecular Probes, Eugene, OR, USA) for 30 min. Additionally, they were incubated with B-HABP at a dilution of 1:100 in PBS containing 1% BSA at room temperature for 1 hr, and then incubated with streptavidin coupled to Alexa (350 nm) for 30 min and mounted on slide glasses with the Vectashield (Vector Lab., Burlingame, CA, USA). Other cryosections were pretreated with phospholipase A_2 (PLA_2) (Sigma, St Louis, MO, USA) at a concentration of 0.01 U/ml in 0.1M Tris buffer and 10 mM $CaCl_2$ at 37 °C for 30 or 60 min.

Double immunofluorescence labeling for PC and FN

Some cryosections were routinely blocked with 10% BSA for 10 min and then incubated with the anti-PC antibody, as described before. Subsequently, they were incubated with the secondary anti-mouse IgM antibody labeled with FITC (594 nm) (Molecular Probes, Eugene, OR, USA) for 30 min, and then with the rabbit anti-FN antibody at a dilution of 1:100 in PBS containing 1% BSA at room temperature for 1 hr. Finally, they were incubated with another secondary anti-rabbit IgG antibody labeled with Alexa (350 nm) (Molecular Probes, Eugene, OR, USA) and mounted on slide glasses with the Vectashield. Other cryosections were pretreated with PLA_2 at a concentration of 0.01 U/ml with 10 mM $CaCl_2$ at 37 °C for 60 min.

Results

Quick-freezing method and immunogold labeling for light microscopy

By the quick-freezing method, articular structures of

TMJ and synovial fluid were well preserved, as expected (Fig. 1a). Silver-enhanced immunogold reaction products for PC were found to be localized in the lower joint cavity (Fig. 1b). Some of them were also localized in a line along the mandibular condylar articular surface tissue (Fig. 1b, inset). Then, exactly same section, corresponding to Figure 1a, was dehydrated in a graded series of ethanol. After such a treatment, the immunogold reaction products were completely abolished (Fig. 1c), suggesting that the PC was easily soluble away from the section on the slide glass by the alcohol-dehydration treatment.

Double immunofluorescence labeling for PC and HA

Some PC immunoreaction products were similarly observed in the lower joint cavity (Fig. 1d). Moreover, another PC-positive immunoreaction was detected along some parts of the upper surface layer in the mandibular condylar cartilage, coexisting with hyaluronic acid (Fig. 1d). In the serial section pretreated with PLA_2 for 30 min, such PC immunoreaction products dramatically decreased in the lower joint cavity (Fig. 1e), but remained on the mandibular condylar upper surface layer. Other immunoreaction products for PC were also slightly observed on the articular surface of the disk (Fig. 1e). However, after treatment with PLA_2 for 60 min, the PC immunoreaction was abolished in the lower joint cavity (Fig. 1f). On the mandibular condylar upper surface layer, the PC-positive immunoreaction could be still detected, although it was diminished considerably by the PLA_2 treatment. Some remnants of PC immunoreaction products were also observed on the articular surface of the disk (Fig. 1f).

Double immunofluorescence labeling for PC and FN

Abundant PC immunoreaction products were found

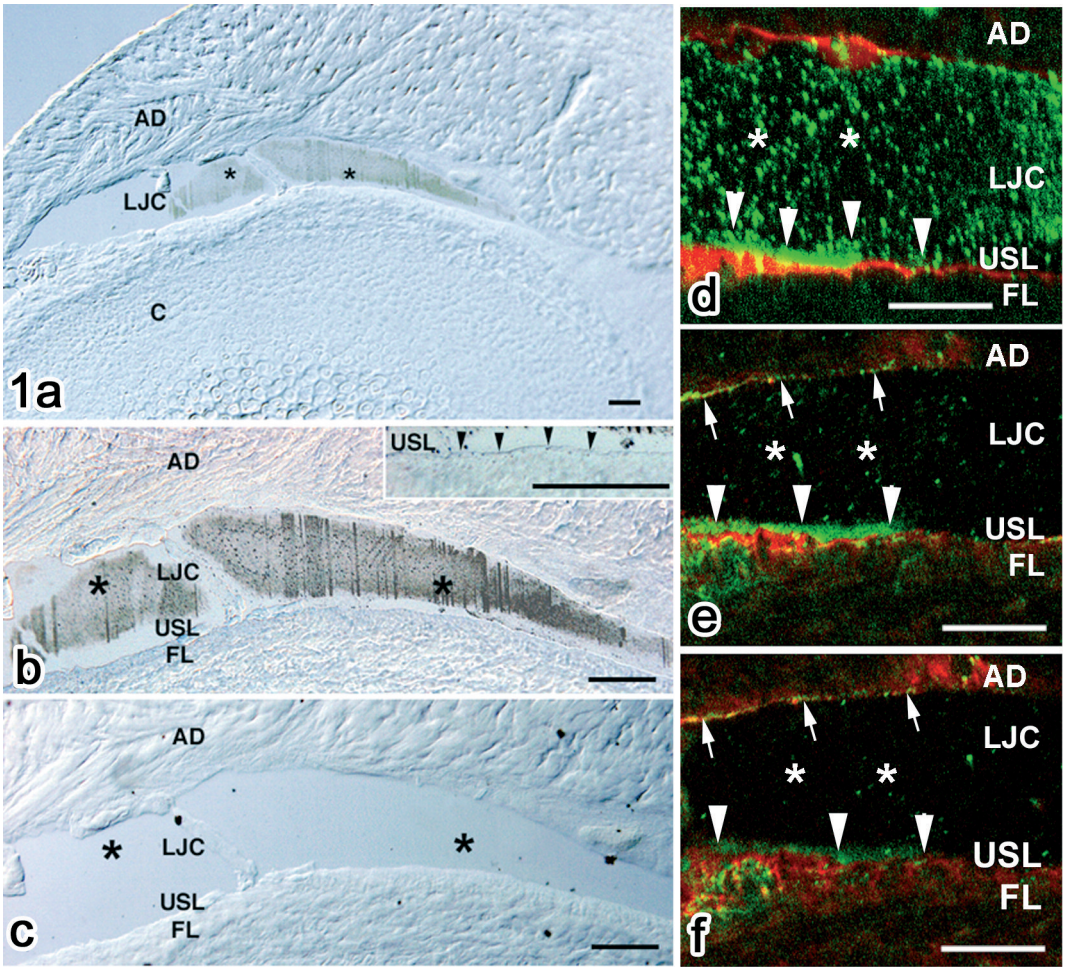

Fig. 1. a-c. Light micrographs of quick-frozen rat TMJ tissues with the immunogold staining for PC. **a.** At low magnification, positive immunoreaction products for PC are localized in the lower joint cavity (asterisks). **b.** Higher magnification of the area shown in (a). Inset; the upper surface layer demonstrating positive immunoreaction products for PC (arrowheads). **c.** The same cryosection shown in (b) after being dehydrated with a graded series of alcohol. Notice that the immunogold reaction was completely abolished (asterisks). **d-f.** Serial sections with double immunostaining for HA (red) and PC (green), as observed by confocal laser scanning microscopy. **d.** The PC is colocalized on the upper surface layer with HA (arrowheads) and on the articular surface of the disk. Another PC-positive immunoreaction is seen in the lower joint cavity (asterisks). **e.** After the treatment with PLA2 for 30 min, the immunoreaction of PC is only observed on the upper surface layer (arrowheads) and the disk (arrows). In the lower joint cavity, the PC immunoreaction was considerably reduced (asterisks). **f.** After the treatment with PLA_2 for 60 min, the PC immunoreaction is almost abolished on the upper surface layer (arrowheads) and the disk (arrows). In the lower joint cavity, the PC immunoreaction is hardly detected. AD: articular disk; LJC: lower joint cavity; C: condyle; USL: upper surface layer; FL: fibrous layer. Scale bars: a-c, 50 μm; d-f, 20 μm.

to be in the lower joint cavity (LJC in Fig. 2a). They were also localized in a line on the upper surface layer of both mandibular condylar cartilage and articular disk (Fig. 2a, arrowheads). On the other hand, other immunoreaction products for FN were localized mainly in the articular surface tissues of both mandibular condylar cartilage (Fig. 2b, arrows) and articular disk. Also, other FN immunoreaction products were observed in the proliferative layer of the mandibular condylar cartilage, as reported before (Zea-Aragon et al., 2004a). In some cryostat sections pretreated with the PLA_2 for 60 min, immunoreaction products for the PC were almost abolished in the lower joint cavity (Fig. 2c). However, articular surface tissues of both mandibular condylar cartilage and articular disk exhibited still clear PC immunoreaction products (Fig. 2d, arrows). Concerning FN immunoreaction, its localization remained on the sections pretreated with PLA_2, as already described above. These findings indicate that some amounts of PC in the upper surface layer may be decorated with some structural components and protected from the PLA_2 digestion treatment.

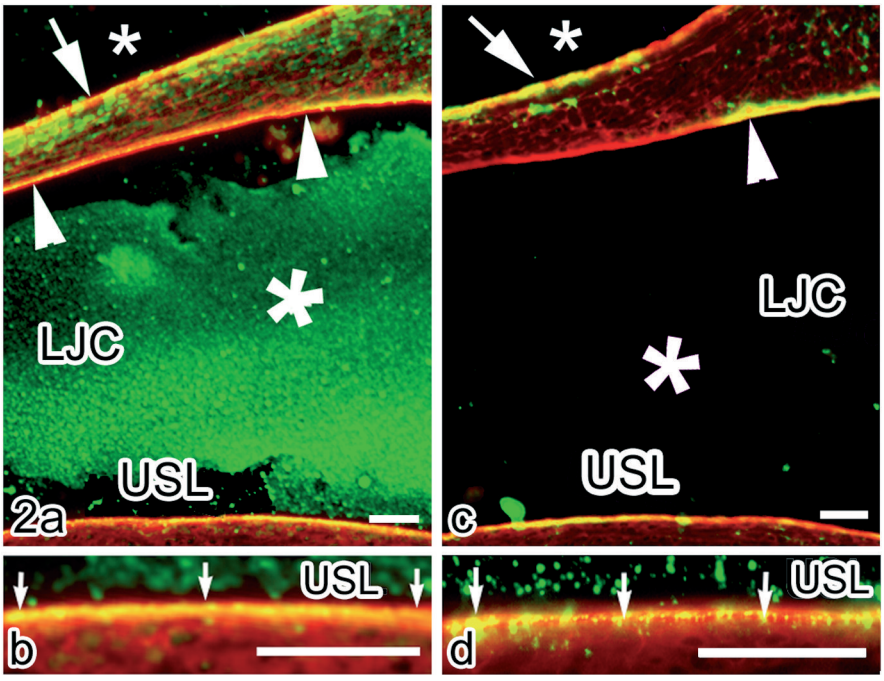

Fig. 2. Double immunofluorescence localizations of PC and FN in the rat TMJ. **a.** The PC (green) is abundantly immunolocalized in the lower joint cavity (large asterisks) and colocalized with FN (red) on the articular surfaces of the disk in the lower (arrowheads) or upper (arrows) joint cavity. Notice that the upper joint cavity has negative immunoreaction for PC (small asterisks) due to washing out after opening the capsule during specimen preparation steps. **b.** Higher magnification of the upper surface layer shown in **(a)**, demonstrating positive immunoreaction products for both PC and FN (arrows)**. c.** A serial section immunostained after the treatment with PLA_2 for 60 min. Such immunostaining for PC is abolished in the lower joint cavity (large asterisks). However, strongly PC-positive immunoreaction is still detected on the articular surface of the disk (arrowheads) and on the surface layer in the upper joint cavity (arrows), colocalizing with FN. **d.** Higher magnification of the upper surface layer shown in **(c),** demonstrating positive immunoreaction products for both remaining PC and FN (arrows). UJC: upper joint cavity; LJC: lower joint cavity; USL: upper surface layer. Scale bars: 50µm.

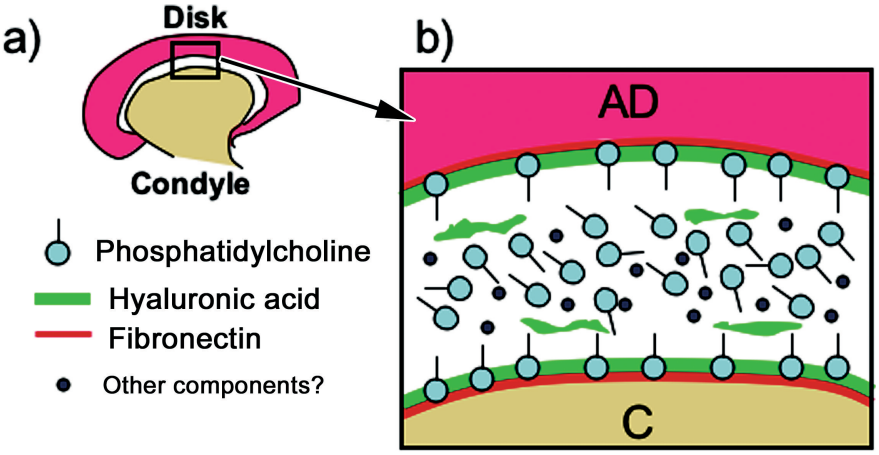

Fig. 3. a. Schematic drawing of the TMJ condyle and disk. **b.** According to our findings, PC is coexisting with hyaluronic acid and fibronectin on the articular surface of disks and condyles as well as in the lower joint cavity, interacting probably with other components. AD: articular disk; C: condyle.

Discussion

Some theories concerning the joint lubrication system, in which both hydrodynamic and boundary lubrications are mainly operative at the articular surfaces, have been extensively reviewed (Hills, 1990; Nitzan et al., 2001). The hydrodynamic lubrication theory postulated that the bearing articular surface was supported by a fluid pressure rather than solid-to-solid contact, being mentioned as "squeeze film" and "weeping bearing" (Lewis and McCutchen, 1959). On the other hand, boundary lubricants are attached to each articular surface, reducing the surface energy for mechanical stresses (Hills, 1989). The surface-active phospholipids (SAPLs; Hills, 1989), in association with a glycoprotein termed as lubricin (Swann et al., 1985), have been assumed to act as an extremely efficient boundary lubricant. The SAPLs are polar lipids which bind to the articular surface with their polar ends, thus orientating their nonpolar moieties outward. The latter imparts a hydrophobic surface function, having a relatively low surface energy, which is much less friction than the articular surface without SAPLs (Hills, 1989, 1990).

The hyaluronic acid (HA)-phospholipid (PL) interactions were examined by ^{13}C-NMR spectroscopy and laser-light scattering methods. In those studies, the chain flexibility of HA in solution was shown to increase due to sonication with dipalmitoyl-phosphatidylcholine (DPPC) (Ghosh et al., 1994). The in vitro formation of HA-PL complexes were also demonstrated by the negative staining and rotary shadowing with electron microscopic techniques (Pasquali-Ronchetti et al., 1997). In the present study, we have examined the PC immunolocalization in the rat TMJ, and compared its co-localization with HA and FN. As shown in Figure 3, we demonstrated that the PC itself was present at the articular surface areas, coexisting with both HA and FN, which were essential to the absorption of phospholipids to the articular surface areas, as also reported with the in vitro studies by others (Ghosh et al., 1994; Pasquali-Ronchetti et al., 1997).

An interesting model of the PC and HA interaction in the TMJ was already proposed (Nitzan, 2001; Nitzan et al., 2001), in which high molecular HA was localized in the joint cavity with PC covering the articular surface. In the present study, our findings showed that the HA was also localized in the articular surface areas and interacted with both PC and fibronectin. Moreover, the fact that the PC was also found to be abundantly present in the lower joint cavity, we now speculate that it may also contribute in some degree to the hydrodynamic lubrication of the TMJ. Considering such findings in the study (Nitzan et al., 2001) and also a previous one (Zea-Aragon et al., 2004a), we consider both HA and FN as principal agents in the boundary lubrication, as illustrated in Figure 3. Although some studies reported that articular specimens treated with hyaluronidase did not increase the friction coefficient (Swann et al., 1974; Hills and Monds, 1998), it is tempting to speculate that the removal of HA on the articular surface during a pathological process will affect the absorption of phospholipids, therefore resulting in an increase in the coefficient friction of the articular surface. Moreover, we demonstrated that the PC immunoreaction in the specimens treated with PLA2 for 30 or 60 min was only slightly diminished on the articular surface of disks and condyles, suggesting that both HA and FN may have a role in the protecting PC from the PLA_2 enzyme digestion.

Concerning the antibody probe for detecting the PC used in the present study, it was highly specific to PC and had no cross-immunoreaction with other phospholipids (Nam et al., 1990). Moreover, it was already successfully used for detection of phospholipid distribution on lipid membranes by a freeze-fracture replica method (Fujimoto, 1995). Recently, we also reported an immunohistochemical study on replica membranes immunolabeled for HA and FN in the rat mandibular condylar cartilage by a quick-freezing method (Zea-Aragon et al., 2004b). In that study, we have clarified some native ultrastructures of the articular upper surface layer in the rat mandibular condylar cartilage. The use of immunohistochemical techniques on the replica membranes provided us with a three-dimensional image of localizations of HA and FN. Therefore, we are now doing further studies on the upper surface layer by using the anti-PC antibody, in combination with the quick-freezing method.

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