Anatomical study of the human discomallear ligament using cone beam computed tomography imaging and morphological observations

By

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Summary: In the present study, the human discomallear ligament (DML) was observed in structures at both macroscopic and cone beam computed tomography levels. Assessments were made regarding the distribution of calcitonin-gene-related peptide (CGRP), protein gene-product (PGP) 9.5, and substance P (SP) of the DML based on immunohistochemical analyses of the anatomical properties of jaw movements using 27 Japanese human cadavers (mean, 79.3 ± 8.6 years; male, 74.9 ± 8.0; female, 82.8 ± 7.5). The DML of the anterior region was connected to the TMJ disc. The DML of the posterior region was attached to both the head and the anterior process of the malleus through the petrotympanic fissure, which formed a narrow channel. The structure of the petrotympanic fissure through the DML was attached to the malleus, and this structure was associated with the mobility of the malleus. In the anterior and posterior parts of the disc-associated connective tissue of the DML, CGRP-, PGP9.5- and SP-positive nerve fibers were located around numerous blood vessels, a condition which may be correlated with chronic pain syndromes disorders and the auditory system.

Introduction

The discomallear ligament (DML) (anterior malleus ligament) and malleo-mandibular ligaments (MMLs) attach to the malleus (Couly and Hureau, 1976; Pinto, 1962). The DML and anterior ligament of the malleus (AML) run through the petrotympanic fissure to enter the TMJ cavity and are attached to the posterior region of the TMJ disc. Then the AML runs from the petrotympanic fissure to the Sphenomandibular ligament (SML) (Burch, 1970; Toledo-Filho et al., 1985; Komori et al., 1986; Sencimen et al., 2008). The attachments of the SML, AML and DML to the malleus are differ (Toledo-Filho et al., 1984; Rodriguez-Vázquez et al., 1998; Abe et al., 1997; Ramirez, 2007). However, the detailed structure of the TML between the petrotympanic fissure and the tympanic cavity is unknown. The DML is a part of the anterior ligament of the malleus (Cesarani et al., 1991; Toledo-Filho et al., 1985) but is also distinguished from the anterior ligament (Oğütçen-Toller, 1995) and these collagen bundles that contain elastic fibers are found in the DML (Sencimen et al., 2009). The mobility of the DML as it passes through the petrotympanic fissure is related to its structure (Couly and Hureau, 1976; Cesarani et al., 1991; Sato et al., 2008). An anatomical relationship between the auditory ossicles and the DML is one of the clinical causes of hearing loss and temporomandibular joint (TMJ) disorders (Gelb et al., 1967; Bernstein et al., 1969; Düké et al., 1972; Pinto, 1962; Ioannides and Hoogland, 1983; Rohlin et al., 1985; Loughner et al., 1989). A relationship between the DML, tympanic cavity and mandibular fossa was also unknown in spite of previous reports for the tympanic cavity using CT images (Petrus and Lo, 1997; Kurosaki et al., 1998; Lacout et al., 2005).

Substance P (SP) and calcitonin-gene-related peptide (CGRP) are related to the afferent nerves, which transmit stimulation to the nucleus as perception fibers of pain (via neurotransmitters). SP- and CGRP-positive nerve fibers exist at the attachment of the lateral pterygoid muscle and the TMJ disc (Johansson, 1986; Ichikawa, 1989, Kido, 1993, Shimizu, 1996), and the distribution of these markers differ in the TMJ disc (Tahmasebi-Sarvestani, 1997, 2001). Previous reports have described the vasculature and nerve structures of the side of the lateral pterygoid muscle (Griffin and Shape, 1960; Castelli, 1963; Sarnat and Engel, 1951; Parsons and Boucher, 1966). However,
the distribution of CGRP, PGP 9.5 and SP in the DML is yet unknown. CGRP-immunoreactive nerves were discovered in the mucosa of obstructive sleep apnea patients (Friberg et al., 1997). PGP9.5 is general neuroendocrine marker protein (Ian and Thompson, 2010). SP concentration is related to chronic pain syndromes (Fischer et al., 1998). The anatomical observations may be related to TMJ disorders and the auditory system. Accurate knowledge of the distribution of PGP9.5, SP and CGRP will be useful to discriminate between auditory and TMJ disorders. Thus, the present study focused on the DML in morphological structures at macroscopic, histological and cone beam CT levels. The study also assessed the neuronal structures within the distribution of SP and CGRP in the DML using immunohistochemical analysis.

Materials and Methods

In this study, temporal bones containing the tympanic cavity and mandibular fossa were examined from 27 human cadavers (mean, 79.3 ± 8.6 years; male, 74.9 ± 8.0; female, 82.8 ± 7.5). All cadavers had been donated for human dissection. Samples were injected with 10% formalin with return perfusion through the femoral artery. After anatomical dissection, the temporal bones with TMJ were removed from the samples.

CT Image

Using cone beam CT, PSR 9000N (Asahi Roentogen Industry, Kyoto, Japan), TMJ images were acquired for each specimen. The cone beam scans were operated at a tube potential of 80 kV and a tube current of 6 mA for 20 sec. and were acquired in cylindrical areas FOV (field of view, in diameter) of Ø41 × 40 mm with high resolution (voxel size 0.1 × 0.1 × 0.1 mm³). A three dimensional structure of the temporal bone with the CT image was reconstructed using a cone beam CT apparatus. The diameter of the mandibular fossa was measured using the software ASHAHI Vision (Asahi Roentogen Industry, Kyoto, Japan). The CT examination was performed under the same geometric conditions and radiological settings as those in routine examinations of TMJ.

Measurements

The measurement points shown in Fig. 1 are based on the CT images of the human TMJ. We measured seven measurements for the mandibular fossa as follows:

a, The length from the center of the articular tubercle to the midline of the posterior edge of the sheath of the styloid process parallel to the ANS line.

b, The width from the spine of the sphenoid to the maximum lateral mandibular fossa.

c, Maximum depth from the ANS palate to the petrotympanic fissure.

d, The largest width of the petrotympanic fissure.

e, The width of the petrotympanic fissure at the midpoint.

f, The width of the petrotympanic fissure at the mandibular fossa.

g, The distance from the entrance of the petrotympanic fissure to the malleus.

Volume of the mandibular fossa; \( v_1 = \frac{4}{3}\pi (a \cdot b \cdot c/2)/2 \)

Volume of the canal of the petrotympanic fissure; \( v_2 = \pi \left\{ \left( \frac{d}{2} \right)^2 + 4\left( \frac{e}{2} \right)^2 + \left( \frac{f}{2} \right)^2 \right\} g \} / 6 \) (see Fig. 1).

Immunohistochemical Methods

Whole mount immunohistochemistry
The DML and the TMJ disc (n = 8) were washed with distilled water for 24 hr, incubated with 3% \( \text{H}_2\text{O}_2 \), for 20 min to eliminate endogenous peroxidase activity and digested with 0.02% proteinase K (Wako, Tokyo, Japan) for 1 hr at 38°C. After overnight fixation in 4% paraformaldehyde, the samples were washed with distilled water for 50 min, and the proteinase K digestion and overnight fixation steps were repeated. The samples were then washed with phosphate-buffered saline (PBS) for 30 min, sequentially incubated in 2.5%, 5% and 10% sucrose in PBS and then subjected to three freeze/thaw cycles. After overnight incubation with 2% Triton X-100 in PBS at 4°C, the samples were washed three times with PBS for 1 hr and incubated for 1 hr at room temperature with 2% normal goat serum/PBS (pH 7.2) containing 0.05% Tween 20 to prevent non-specific antibody binding. The samples were then incubated with rabbit polyclonal antibodies against CGRP diluted (1 : 1000; Biogenesis, NH, USA), PGP9.5 (diluted 1 : 100; Lab Vision, CA, USA), and SP (diluted 1 : 100; Lab Vision, CA, USA) or normal goat serum as a negative control. Samples were washed three times with PBS for 1 hr. Following the manufacturer’s instructions, sections were incubated with HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, USA). The samples were then washed three times with PBS for 1 hr. The staining was visualized using 0.02% \( \text{H}_2\text{O}_2 \) and 0.1% (1 mg/ml) diaminobenzidine tetrahydrochloride in 0.1 M Tris-HCl, pH 7.2. Images were acquired using a stereomicroscope (Leica MZ 16FA; Leica Microsystems, USA) with the Leica Application Suite software (Leica Microsystems).

Immunohistochemistry to tissue section
The DML were embedded into OCT (Tissue Tek II, Sakura Finetechinical Co., Ltd, Tokyo, Japan) compound, frozen in liquid nitrogen and stored at −80°C until use. Frozen sections (about 10 μm thick) of the samples were
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Cut and fixed for an additional 10 min in a solution of 10% paraformalin. These sections were incubated with 50-fold diluted normal goat serum in PBS (pH 7.2, 0.05% Tween 20) in order to prevent subsequent non-specific absorption of antibodies for 1 hr at room temperature. After separate incubation with antibodies against Fibronectin (diluted 1:100; LB-1027, LSL, Japan) and Tenascin C (diluted 1:200; ab465, Abcam Ltd, Cambridge,

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Fig. 1. Measurement points of the human TMJ by CT image. The mandibular fossa in Norma basalis of dry skull (A, bar = 0.5 cm): Sagittal section of temporal bone of cone beam CT image in the human TMJ (B, bar = 1 cm), incus; M, malleus bone; MF, mandibular fossa.

- a, The length from the center of the articular tubercle to the midline of the posterior edge of the sheath of the styloid process parallel to the ANS line
- b, The width from the spine of the sphenoid to the maximum lateral mandibular fossa
- c, Maximum depth from the ANS palate to the petrotympanic fissure
- d, The largest width of the petrotympanic fissure
- e, The width of the petrotympanic fissure at the midpoint
- f, The width of the petrotympanic fissure at the mandibular fossa
- g, The distance from the entrance of the petrotympanic fissure to the malleus
UK), CGRP (diluted 1:1000; Biogenesis, NH, USA), PGP9.5 (diluted 1:100; Lab Vision, CA, USA), and SP (diluted 1:100; Lab Vision, CA, USA) or normal goat serum as a negative control. These sections were washed with PBS 5 min × 3 times. Following the manufacturer’s protocol, these sections were incubated with biotinylated goat antibodies against rabbit IgG (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA., USA). Then, the specimens and controls (treated with normal goat serum) were washed with PBS-0.05% Tween 20 PBS 5 min × 3 times, and finally incubated with a mixture of reagents from an elite ABC kit that contained 0.02% H2O2 to identify in the DML. After observation by a light microscope as a negative control. These sections were washed (diluted 1:100; Lab Vision, CA, USA) or normal goat Kit; Vector Laboratories, CA, USA). Then, goat antibodies against rabbit IgG (Vectastain Elite ABC protocol, these sections were incubated with biotinylated staining (HE) and resorcinfuchsin stain for elastic fibers in 0.1 M Tris-HCl, pH 7.2). In addition to general staining, we performed hematoxylin alizarin red S and eosin staining (HE) and resorcinfuchsin stain for elastic fibers to identify in the DML. After observation by a light microscope, Images were acquired using a stereo microscope (Leica DM 2500; Leica Microsystems, USA) with the Leica Application Suite software (Leica Microsystems). At the sagittal section, the petrotympanic fissure varied in size at Reid’s base line according to the CBCT images (Fig. 4). The entrance of the petrotympanic fissure at the tympanic cavity displayed a triangular tunnel structure that became narrow (width, 0.9 ± 0.4 mm). We also classified three types of the structure in the petrotympanic fissure from the mandibular fossa to the tympanic cavity: a tunnel-shaped structure widely open in the entrance of the mandibular fossa (type 1), middle region with flat-shaped tunnel structure (type 2) and narrow exit (type 3) in the tympanic cavity by Sato et al., (2008). The volume of the mandibular fossa and the canal of the petrotympanic fissure increased from narrow exit type to widely open entrance type (see Figs. 5A–C).

Observations and measurements of the tympanic cavity, the petrotympanic fissure and the mandibular fossa using cone beam CT

At the sagittal section, the petrotympanic fissure was located in the lateral-posterior side of the mandibular fossa and formed a narrow channel that was elongated in the anterior direction of the epitympanic recess in the upper part of the tympanic cavity. In this cavity, the malleus and incus bones were observed. At the inferior side of the epitympanic recess was the tympanic cavity, while the compacted bone that constitutes the carotid canal was observed in the posterior wall of the tympanic cavity. The compacted bone structure was also found in a part of the styloid process sheath. The petrotympanic fissure resembled a small lumen-like structure between the epitympanic recess of the tympanic cavity and the mandibular fossa. The position of the malleus bone was located in the inferior part of the epitympanic recess of the tympanic cavity, and the location of the malleus was classified into two types by CT. Type 1 (46.3%; 25/54) involved the malleus being nearly connected to the opening of the petrotympanic fissure, while Type 2 (53.7%; 29/54) involved its being more than 1 mm away from the petrotympanic fissure (Fig. 4). Figure 5 shows the diameter of the petrotympanic fissure and the tympanic cavity at Reid’s base line. The anterior-posterior diameter of the mandibular fossa was 14.9 ± 2.5 mm, and the diameters of the entrance and exit of the petrotympanic fissure varied in size at Reid’s base line according to the CBCT images (Fig. 4). The entrance of the petrotympanic fissure to the mandibular fossa was large (width, 1.9 ± 0.7 mm), while the exit of the petrotympanic fissure at the tympanic cavity displayed a triangular tunnel structure that became narrow (width, 0.9 ± 0.4 mm). We also classified three types of the structure in the petrotympanic fissure from the mandibular fossa to the tympanic cavity: a tunnel-shaped structure widely open in the entrance of the mandibular fossa (type 1), middle region with flat-shaped tunnel structure (type 2) and narrow exit (type 3) in the tympanic cavity by Sato et al., (2008). The volume of the mandibular fossa and the canal of the petrotympanic fissure increased from narrow exit type to widely open entrance type (see Figs. 5A–C).

Ethics

The human cadavers were obtained from donation system using the guidelines from the Law Concerning Body Donation for Medical and Dental Education (the Body Donation Law) and the Law Concerning Cadaver Dissection and Preservation (LCCDP). All of the samples were examined from Nippon Dental University Collections.

Statistical Analysis

Differences in the frequency of the samples with a given reactivity level among the groups were analyzed using the Kruskal-Wallis one-way analysis of variance with a post hoc Tukey’s test. A p value of less than 0.05 was considered statistically significant. Pearson’s correlation coefficient was used to measure the strength of the association between those two variables.

Results

Macroscopic observation of the discomalleolar ligament and the TMJ disc

The discomalleolar ligament (DML) was arranged from the upper-posterior region of the mandibular fossa of the petrotympanic fissure to the anterior region of the malleus in the epitympanic recess of the tympanic cavity. The DML was connected to the TMJ disc (Figs. 2A, 2B). Under high magnification, it was observed that this fascicle covered the malleus. The chorda tympani (CHT) was seen parallel to the TML and slightly downward. The anterior ligament of the malleus (AML) was found in the lateral-inferior part of the malleus. The tensor tympani ran parallel to the auditory tube, and shifts in the DML were seen in the sphenomandibular ligament (SL) in the lower part of this auditory tube. The DML was attached to both the head and the anterior process of the malleus while the AML originated from inside the head of the malleus. In addition, the chorda tympani ran along the basal region of the malleus (Figs. 3A–C).
Distribution of nerve fibers (anti-CGRP, PGP9.5 and SP positive fibers) and the extracellular matrices

In the anterior and posterior parts of the disc-associated connective tissues of the DML at macroscopic levels, CGRP-, PGP9.5-, and SP-positive nerve fibers were located around numerous blood vessels (Fig. 6A–F). We also observed, using alizarin red S, numerous blood vessels in the same parts of the disc-associated connective tissues. Smaller branches of these positive nerves were scattered in the disc membranes of the anterior and posterior regions of the discs (Fig. 6). However, these positive fibers were not detected in the mid-portion of the TMJ disc. In the disc side of the DML, numerous CGRP-, PGP9.5- and SP-positive nerve fibers were also found. These numerous positive fibers were scattered around the vessels (Figs. 6A–H). In the longitudinal section of the DML, fine positive fibers were found in the vascular walls and connective tissues near small vessels (Figs. 7A–D).

The numerous elastic fibers forming a shrinkage structure were found in the connective tissues of the DML (8.8% ± 1.04 per 100 µ square). These fibers were...
Fig. 3. Lateral view of left tympanic cavity. (male 85 years old)

A, Lateral view of the left mandibular disc and tympanic cavity (Bar = 4 mm).

B, The discomallear ligament (DML), anterior malleus ligament (AML), and chorda tympani (CHT) are found under the auditory tube (AT) and tensor tympani (TT) (Bar = 2.5 mm).

C, The DML is appeared in the temporal bone containing petrotympanic fissue (Bar = 2.5 mm).

Disc; articular disc of temporomandibular joint; HM, head of malleus; I, incus; M, malleus; SML, sphenomandibular ligament
mainly located in the central region of the DML bundle (Fig. 8E, F). Numerous positive reaction fibers of fibronectin (18.3% ± 1.44 per 100 μ square) and tenascin C (17.37% ± 1.48 per 100 μ square) were also found in the connective tissues of the DML (Fig. 8D), although no specific distribution was found and they were ubiquitously scattered around the connective tissues (Figs. 8A–C).

Discussion

TMJ disorder was caused the specific morphology of the TMJ disc connected with the discomallear ligament (DML) and the anterior malleus ligament (AML) (Ioannides and Hoogland, 1983; Loughner et al., 1989; Ogutcen-Toller and Juniper, 1993; Cheynet et al., 2003). Previous reports have shown that the attachment of the DML becomes narrower toward the malleus. Kim et al. (2004) defined three classifications: direct; indirect; and both direct and indirect. However, this classification is not sufficient in defining the structure of the DML because these reports did not show anatomical figures. In our study, we used CBCT images to classify two types according to the location of the malleus in the epitympanic recess of the tympanic cavity. Our observations indicated two types: type 1, which is located in the malleus adjacent to the opening of the petrotympanic fissure (46.3%), and type 2, which is located (53.7%) more than 1 mm away from the petrotympanic fissure. The malleus connected with the TML in Type 2 easily moves freely by the TMJ movenets. In this case, the tension of the TMJ disc during opening jaw may affect the auditory system. Moreover, the anatomical structure of the hard tissues is related to the mobility of the DML as it passes through the petrotympanic fissure (Coulé and Hureau, 1976; Cesarani et al., 1991; Sato et al., 2008). Kim et al. (2004) reported that all examined cases (n = 16) moved according to the investigation of overstretched using forceps. In our cases, the structure in the petrotympanic fissure from the mandibular fossa to the tympanic cavity is classified type 1; a tunnel-shaped structure widely open in the entrance of the mandibular fossa. The DML easily moves in this case as it passes through the petrotympanic fissure.

In the posterior region of the TMJ disc, numerous elastic fibers (Clément et al., 2006) are connected to the DML. In our study, the elastic fibers were found in the anterior region of the DML, connected to the posterior region of the TMJ disc. The DML is stretched from tensions in the TMJ disc during jaw opening. Moreover, numerous extracellular matrices such as fibronectin and tenascin C were also found in this region. Tenascin C is an adhesion-modulating protein (Chiquet-Ehrismann, 1993). It is an elastic protein that is connected to fibronectin and is important in the formation and arrangement of the collagen fibers. The load-induced expression of tenascin C has been found in tendons (Jarvinen et al., 2000). In our study, tenascin C and fibronectin were widely arranged in the DML along with the collagen fibers, although without any no localization in the region. It is suggested that this thin bundle of DML is an elastic structure with tension.

Fig. 4. Sagittal section of Temporal bone of cone beam CT image in the human TMJ.

The position of the malleus bone was located in the inferior part of the epitympanic recess of the tympanic cavity. The location of the malleus was classified into two types by CT. Type 1 (A) involved the malleus being nearly connected to the opening of the petrotympanic fissure. Type 2 (B) involved its being more than 1 mm away from the petrotympanic fissure.
Fig. 5. The measurement data shown in A, B, and C are based on the CT images of the human TMJ.

A. Four measurement points (d, e, f, g) data for the temporal bone (see Figure 1).

B. Volume of the mandibular fossa; $V_1 = \frac{4}{3}\pi (a \cdot b \cdot c/2)/2$

C. Volume of the canal of the petrotympanic fissure; $V_2 = \pi [((d/2)^2 + 4(e/2)^2 + (f/2)^2)g] + 6$ (see Figure 1).

A tunnel-shaped structure widely open in the entrance of the mandibular fossa (type 1), middle region with flat-shaped tunnel structure (type 2) and narrow exit (type 3) in the tympanic cavity by Sato et al., (2008).

- d, The largest width of the petrotympanic fissure.
- e, The width of the petrotympanic fissure at the midpoint.
- f, The width of the petrotympanic fissure at the mandibular fossa.
- g, The distance from the entrance of the petrotympanic fissure to the malleus. *, p < 0.05; **, p < 0.01; ***, p < 0.001.
Fig. 6. Whole mount immunohistochemical reaction of nerve fibers (anti-CGRP, PGP9.5 and SP positive fibers) on the posterior region of the connective tissue (A, C, E, G) of the articular disc of temporomandibular joint and discomallear ligament (B, D, F, H).

A, CGRP (Bar = 250 μm); B, CGRP (Bar = 250 μm); C, SP (Bar = 250 μm); D, SP (Bar = 200 μm); E, PGP9.5 (Bar = 1 mm); F, PGP9.5 (Bar = 500 μm); G, alizarin red S (Bar = 250 μm); H, alizarin red S (Bar = 1 mm).
capacity caused by stretches to the TMJ disc during jaw movements. The composition of the elastic fibers and the extracellular matrices (e.g., tenascin C and fibronectin) are also finely controlled by tension from the posterior region of the connective tissues in the TMJ disc during jaw opening.

Numerous nervous fibers and fine blood vessels are located in the posterior region of the TMJ disc (Thilander, 1961; 1964). The distribution of the nervous fibers forming a free nerve ending is altered during development (Thilander, 1964). In our study, anti-SP-, PGP-, and CGRP-positive fibers were also found in the posterior region of the TMJ disc. Previous reports have indicated that anti-SP- and CGRP-positive fibers were found in the attachment of the lateral pterygoid muscle to the TMJ disc (Johansson, 1986; Ichikawa, 1989, Kido, 1993, Shimizu, 1996). The specific location of the markers is related to the function of the sensory receptor of control for excess jaw movements or tension of the TMJ disc. These markers were also found in the patellar ligament and Achilles tendon (Bjur et al., 2005; Andersson et al., 2007). These ligaments withstand continuous stresses, such as pressure or tension from muscle movements. Therefore, the SP-, PGP-, and CGRP-positive fibers, such as the afferent nerves that act as perception fibers for pain, are important indicators. The structure of the petrotympanic fissure through which the discomalleolar ligament attaches to the malleus, as well as the histological and radiological profiles of its structure, is related to the mobility of the malleus.

Fig. 7. Immunohistochemical reaction of nerve fibers (anti-CGRP, PGP9.5 and SP positive fibers) in the discomalleolar ligament.
A, CGRP (Bar = 50 μm); B, SP (Bar = 30 μm); C, PGP9.5 (Bar = 25 μm); D, contral (Bar = 100 μm)
Fig. 8. Immunohistochemical reaction of extracellular matrices (fibronectin, tenascin C), hematoxylin and eosin staining (HE) and resorcinfuchsin stain in the discomalleolar ligament.

A, Fibronectin (Bar = 100 μm); B, Tenascin C (Bar = 200 μm); C, control (Bar = 200 μm); D, composition of the fibronectin, tenascin C, and elastic fibers (per square of 100 μm) of each section (EF, elastic fiber, FN, fibronectin, TNC tenascin C fibers); E, HE (Bar = 1 mm); F, resorcinfuchsin stain (elastic fiber) (square is a large magnification of the fig. 8F) (Bar = 1 mm)
References


