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Expression of chondromodulin-1 in the temporomandibular joint condylar cartilage and disc

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Abstract

BACKGROUND:

The temporomandibular joint (TMJ) cartilage consists of condylar cartilage and disc and undergoes continuous remodeling throughout post-natal life. To maintain the integrity of the TMJ cartilage, anti-angiogenic factors play an important role during the remodeling process. In this study we investigated the expression of the anti-angiogenic factor, chondromodulin-1 (ChM-1), in TMJ cartilage and evaluate its potential role in TMJ remodeling.

METHODS:

Eight TMJ specimens were collected from six 4-month-old Japanese white rabbits. Safranin-O staining was performed to determine proteoglycan content. ChM-1 expression in TMJ condylar cartilage and disc was determined by immunohistochemistry. Three human perforated disc tissue samples were collected for investigation of ChM-1 and vascular endothelial growth factor (VEGF) distribution in perforated TMJ disc.

RESULTS:

Safranin-O stained weakly in TMJ compared with tibial articular and epiphyseal cartilage. In TMJ, ChM-1 was expressed in the proliferative and hypertrophic zones of condylar cartilage and chondrocyte-like cells in the disc. No expression of ChM-1 was observed in osteoblasts and subchondral bone. ChM-1 and VEGF were both similarly expressed in perorated disc tissues.

CONCLUSIONS:

ChM-1 may play a role in the~~is a putative~~ regulat~~ion~~~~or of~~~~in~~ TMJ remodeling~~,~~ ~~by~~~~acting~~ ~~by~~ prevent~~ing~~~~ing~~ blood vessel invasion of the cartilage,~~,~~ ~~th~~~~ereby~~~~us~~ maintaining condylar cartilage and disc integrity.

Introduction

Temporomandibular joint (TMJ) remodeling is physiologically a complicated procedure, which involves both chondrogenesis and osteogenesis (1, 2). The TMJ, which includes the condylar cartilage and TMJ disc, is a unique structure and differs from other synovial joints in that it requires constant remodeling throughout life. TMJ condylar cartilage remodeling is related to mandibular growth and in adaption to occlusion movement, as well as load bearing during masticating and some pathological changes such as disc perforation (3, 4). Angiogenesis is a crucial step in TMJ remodeling, as it regulates the transformation from cartilage to bone (5). Angiogenesis is accompanied by the expression of angiogenic factors such as vascular endothelial growth factor (VEGF), which involves the formation of new blood vessels from pre-existing vessels to form a capillary networks. During long bone growth, VEGF is expressed in hypertrophic chondrocytes leading to the recruitment of endothelial cells into the cartilage with subsequent osteogenic induction (6). VEGF expression has been reported in the hypertrophic zone of healthy TMJ condylar cartilage during remodeling (7). This is paradoxical given that cartilage is an avascular tissue and angiogenesis in cartilage is detrimental to the maintenance of its integrity, a condition which is typically the forerunner of osteoarthritis (8). The healthy TMJ disc is subject to remodeling, as is condylar cartilage (9). Therefore, angiogenic and antiangiogenic factors need to be well regulated to maintain the TMJ and disc integrity. However, the molecular mechanisms, underlying the maintenance of the structural integrity during TMJ remodeling, remains unclear.

ChM-1, a 25-kDa glycoprotein, is an anti-angiogenic factor and has been purified from articular cartilage. ChM-1 has been found to be strongly expressed in immature cartilage and prevents blood vessels growing into the cartilage. It has been suggested that ChM-1 is responsible for maintaining the cartilage integrity during osteogenesis (10). TMJ cartilage is classified as a fibrocartilage and differs from other synovial joints cartilage by expressing type I rather than type II collagen (11). Previous studies have tended to focus on the angiogenic factors in TMJ remodeling (12), whereas little is known of the anti-angiogenic factors in TMJ. In this study we investigated, by immunohistochemistry, the spatial distribution of ChM-1 in TMJ condylar cartilage and disc.

Materials and Methods

This study was carried out according to the guideline of the Animal Ethics Committee at School of Stomatology, Wuhan University. A total of eight temporomandibular joints (five right sides, three left sides) were harvested from six healthy 4-month-old Japanese white rabbits; the animals weighing approximately 2kg each. Four samples harvested from the tibia of adult female Lewis rats served as positive controls. Three perforated TMJ disc tissues were obtained from human volunteers undergoing TMJ disc surgery after informed consent was given. Except for the human samples, all the other samples were removed and fixed in 4% paraformaldehyde for 48 hr, decalcified in a 10% ethylenediaminetetraacetic acid disodium (EDTA-2Na) solution

for 4 weeks at 4℃, and then embedded in paraffin.   Serial sagittal sections (5µm) of the paraffin blocks were cut with a Leica RM2265 microtome (Leica, Wetzlar, Germany).   The sections were dewaxed in xylene and then hydrated in gradual decreased ethanol concentrations.   For Safranin-O staining, sections were first stained with Weigert's haematoxylin (HT107 & HT109, Sigma-Aldrich, St. Louis, MO, USA) for 10 min, dedifferentiated with acid alcohol, then stained with fast green and Safranin-O for 5 min respectively.   The sections were washed and dehydrated in gradual increased ethanol and cleared in xylene, and finally mounted with Depex mounting medium (BDH Laboratory, Poole, UK).

Immunohistochemistry was performed using a goat anti-human ChM-1 antibody, (sc-20310; Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA).   The sections were first incubated with proteinase K for 10 min, and then incubated with 3% H_2O_2 for 20 min to inactivate endogenous peroxidase.   Non-specific binding was blocked with a donkey blocking serum (diluted 1:10 in PBS, sc-2044, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA).   The sections were incubated with ChM-1 antibody (diluted 1:100) in a humidified chamber at 4°C overnight, after which they were washed thoroughly with PBS, incubated with biotin-conjugated donkey anti-goat IgG (diluted 1:100; sc-2042 ,Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), in a humidified chamber for 30 min, at room temperature, and then rinsed thoroughly with PBS and incubated with streptavidin conjugated horseradish peroxidase (DAKO, Carpinteria, CA, USA).   Finally, the bound immunocomplex was visualized by immersing the sections in 3, 3'-diaminobenzidine (Sigma, St. Louis,

MO, USA) for 2 min. Negative controls were provided by replacing the primary antibody with diluted BSA. Rabbit anti-human VEGF antibody (RB-220-P1,Thermo scientific, Fremont, CA, USA) was used to detect the expression of VEGF in human disc tissues and followed the same protocol as above, but excluding proteinase K incubation. Sections were counterstained with Mayer's haematoxylin (Sigma, St. Louis, MO, USA), and mounted with Depex mounting medium.

Safranin-O staining, ChM-1 and VEGF immunoreactivity were independently evaluated by two experienced researchers and the intensity of staining was assessed and graded as either no expression, weak, moderate, or strong expression.

Results

Safranin-O staining

In the tibia, positive Safranin-O staining extended throughout the full thickness of articular and epiphyseal cartilage (Fig.1a and b). Staining was absent in the superficial zone of articular cartilage and subchondral bone (Fig.1a and b, table 1).

In the TMJ, positive Safranin-O staining was weaker in condylar cartilage and disc, compared to staining found in the tibia samples (Fig.1c and d, table 1). The matrix of disc stained very weakly (Fig.1c) and the only positive staining was associated with chondrocyte-like cells (Fig.1c). Positive staining was noted in the proliferative and

hypertrophic zone in condylar cartilage (Fig.1d), whereas no staining was found in fiber zone of condylar cartilage (Fig.1d, table 1).

ChM-1 immunostaining.

In the tibial articular cartilage the expression of ChM-1 was faint and found around the chondrocytes, with no obvious staining in cartilage matrix (Fig.2a).   ChM-1 was expressed in the resting, proliferating and hypertrophic zones of epiphyseal cartilage and found both in chondrocytes and cartilage matrix (Fig.2b).   There was no ChM-1 immunoreaction in the subchondral bone.

In the rabbit TMJ samples, ChM-1 expressed strongly in the cytoplasm of chondrocyte-like cells in anterior, intermediate and posterior bands and bilaminar zones throughout the disc, except for the superficial layer (Fig.2c, table 1).   ChM-1 expression was predominantly located in the proliferative and hypertrophic zone of condylar cartilage and the immunoreactions was in the cytoplasms of ChM-1 positive cells (Fig.2d).   There was an absence of ChM-1 staining in the fiber zone and in the matrix of the disc (Fig.2c), subchondral bone of TMJ (Fig.3), and in the synovium.

ChM-1 and VEGF expression in Human perforated disc samples

In human perforated disc samples, ChM-1 expression was noted in most chondrocyte-like cells (Fig 4a).   A similar distribution of VEGF was also detected in all the patient's samples collected from the perforated TMJ discs (Fig 4b).

Discussion

TMJ cartilage, which includes the condylar cartilage and disc, is a fibrocartilage which undergoes remodeling in response to external stimuli. Previous studies have found that the amount of proteoglycan in TMJ cartilage is relatively lower than that found in other synovial joint cartilage (13). In our study we found that the positive staining of safranin-O was weaker in TMJ condylar cartilage than in tibia articular and epiphyseal cartilage. The safranin-O staining was restricted to the areas surrounding of chondrocyte-like cells in the disc, which is evidence of TMJ remodeling.

TMJ cartilage is surrounded by an angiogenic microenvironment with the expression of VEGF in the hypertrophic zone of healthy TMJ condylar cartilage (7). An angiogenic microenvironment typically leads to blood vessel invasion and a loss of cartilage matrix (12, 14). An earlier study has showed that angiogenesis is involved in cartilage matrix degeneration and osteophyte formation in OA (15). It is therefore a reasonable hypothesis that antiangiogenic factors play an important regulatory role in maintaining healthy TMJ cartilage integrity. ChM-1 is a specific anti-angiogenic factor isolated from cartilage (16), and is strongly expressed in growing articular cartilage, epiphyseal cartilage, and normal adult joints, playing a role in the maintenance of avascularity in articular cartilage (10, 17-19). Its presence modulates the angiogenic effect of hypoxia (20) and inhibits DNA synthesis and proliferation of vascular endothelial cells, as well as *in vitro* tube morphogenesis (21). During the growth period, ChM-1 is expressed throughout articular and epiphyseal

cartilage to modulate endochondral ossification, a process similar to the remodeling in TMJ. ChM-1 expression decreases in mature articular cartilage, but is still expressed in epiphyseal cartilage after growth (10). An *in vitro* study found that, in response to differentiation stimuli, *ChM-1* mRNA expression in primary cultured chondrocytes was markedly down-regulated (22). An absence of ChM-1 creates a microenvironment which favors blood vessel invasion into cartilage (23). However, the role of ChM-1 in TMJ cartilage is unclear and has not been subject to investigation.

In this study, we provide evidence for the expression of ChM-1 in adult TMJ condylar cartilage. ChM-1 was strongly expressed in TMJ condylar cartilage in the proliferative and hypertrophic zones, a front line to blood vessel invasion. This expression profile was similar to ChM-1 expression in epiphyseal cartilage but different to the distribution in articular cartilage (10), which indicates that angiogenic properties between TMJ cartilage and articular cartilage are different. VEGF has been identified in healthy condylar cartilage and here the presence of ChM-1 could act as a barrier which prevents blood vessel invasion into the cartilage. The role of ChM-1 expression in the TMJ condyle may therefore be of a similar nature to that in immature articular cartilage and epiphyseal cartilage, suggesting that ChM-1 regulates TMJ remodeling, thus maintaining TMJ cartilage integrity.

The TMJ disc undergoes a synchronized remodeling together with the TMJ articular cartilage remodeling (24). The TMJ disc is an avascular tissue and our findings

suggest that the existence of ChM-1 in the disc may prevent blood vessel invasion. In TMJ disorders, disc perforation is an important pathological symptom of the disease. The perforated disc does not readily repair by natural wound healing, even though VEGF has been observed in disc chondrocyte-like cells located in disc perforation areas (25). In this study, we found that VEGF was expressed in chondrocyte-like cells of perforated TMJ disc. Interestingly, ChM-1 was also found to be distributed similarly in chondrocyte-like cells. It is therefore possible that the existence of ChM-1 neutralizes the angiogenic effect of VEGF and prevents blood vessel invasion at the arthropathic site thereby retarding perforation healing. The distribution of ChM-1 in fibrocartilage tissue has been reported in intervertebral disc (26) and in the remaining chondrocytes in the degenerative intervertebral disc (27).

TMJ remodeling is a physiologically complicated process which involves both chondrogenesis and osteogenesis. Interaction between angiogenic and anti-angiogenic factors regulates TMJ remodeling, maintaining the avascular property and integrity of TMJ cartilage so that the joint can function normally. Our findings suggest that ChM-1 plays an important role in both condylar cartilage and TMJ disc by maintaining the avascular properties during TMJ remodeling and disc perforation.

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Legends

Figure 1: ~~Representative images of~~ Safranin O staining in cartilage. In tibia, Safranin O staining was intense ~~ive~~ in articular (a) and epiphyseal (b) cartilage. In TMJ, Safranin O staining was faint in disc (c) condylar cartilage (d) and limited to the surrounding of disc cells (c). A: articular cartilage; C: condylar cartilage; D: TMJ disc; E: epiphyseal cartilage; Em: articular eminence; F: the fibrosis zone; H: the hypertrophic zone; P: the proliferative zone; R: the resting zone. Bar = 100μm

Figure 2: ~~Representative images of~~ ~~I~~immunostaining for ChM-1 in cartilage. In tibia, ChM-1 expression was faint in articular cartilage (a) but intense in epiphyseal cartilage (b). In TMJ, ChM-1 expression was limited to cells in the disc and absent in the disc matrix (c) ChM1 staining was intense in condylar cartilage (d). A: articular cartilage; C: condylar cartilage; D: TMJ disc; E: epiphyseal cartilage; F: the fibrosis zone; H: the hypertrophic zone; P: the proliferative zone; R: the resting zone. Bar = 100μm

Figure 3: There was an absence of ChM-1 staining in TMJ condylar subchondral bone. Arrow indicates subchondral bone. Bar = 50μm

Figure 4: ChM-1 (a) and VEGF (b) expression in chondrocyte-like cells in human TMJ perforated disc samples. Bar =50μm Arrows in figures a and b show positive cells in TMJ disc respectively. Bar =20 μm.

Table 1: Staining intensity of Safranin O and ChM-1 in tibia and TMJ cartilage