**Introduction**

Bone morphogenetic protein 3 (BMP3) is a member of the transforming growth factor beta (TGF-β) superfamily, which plays important roles in growth and development. There is a study showing that Bmp3 expression was reduced in bone and cartilage tissues with mechanical loading in vivo. To our knowledge, there are no published reports clearly showing the localization of Bmp3 mRNA in mandibular condylar cartilage and its response to mechanical stress. The present study mapped the distribution pattern of Bmp3 in the mandibular condylar cartilage. In comparison with that in condylar cartilage, we investigated the Bmp3 mRNA expression in two other mechanically loaded cartilages samples, i.e., femoral articular cartilage and temporal cartilage, which transiently appeared in the reparative response stage during mandibular ramus fracture healing. We also investigated one mechanically unloaded cartilage, i.e., femoral growth plate cartilage. To characterize the Bmp3 mRNA-positive chondrocytes, we analyzed the distribution of type I, type II, and type X collagen mRNA expression.

**Materials and methods**

*Fracture model and tissue preparation*  A total of 16 six-week-old male Wistar rats weighing 160-180 g were used. Rats were divided into 2 groups, 12 rats for the fracture experiment and 4 rats as the control group. Surgery was performed on the right side of the mandibular. All surgically treated animals were fed an ordinary diet of rodent feed, administered in powder form for 1 week postoperatively and with regular thereafter. At 3, 7, 14 days post operatively, 4 rats were perfused with 4% PFA in 0.1M sodium phosphate buffer (PH 7.4), than the mandible with surrounding tissue was dissected. The obtained tissue was fixed in 4% PFA at 4°C for 24 hours, and decalcified with 20% EDTA for 2 weeks, then dehydrated before being embedded in paraffin
wax. Longitudinal sections, 7 μm thick, were cut and mounted on triethoxyaminopropylsilane-coated slides. The slides were stored at 4°C until used. Four rats in the control group were treated in the same way but without fracture treatment.

Probe preparation and In situ hybridization Digoxigenin-11-UTP-labeled single-stranded RNA probes were prepared with a DIG RNA Labeling kit according to the manufacturer’s instructions. A 348-bp fragment of Bmp3, a 240-bp fragment of type I collagen, a 300-bp fragment of type II collagen and a 440-bp fragment of type X collagen were used to generate sense and anti-sense probes. Tyramide Signal Amplification Biotin System was used for in situ hybridization according to the manufacturer’s protocol. Controls were hybridized with sense probes.

[Results]

Based on chondrocyte morphology, the mandibular condylar cartilage and femoral articular cartilage were divided into fibrous, proliferating, mature, and hypertrophic cell layers, the femoral growth plate cartilage was divided into four layers: resting, proliferating, mature, and hypertrophic cell layers. In mandibular condylar cartilage, Bmp3 was strongly expressed in the proliferating, mature and hypertrophic cell layers. In femoral growth plate cartilage and femoral articular cartilage, Bmp3 was strongly expressed in proliferating, mature and upper hypertrophic chondrocytes in femoral articular cartilage, whereas it was sparsely distributed in hypertrophic chondrocytes of the epiphyseal growth plate cartilage. In the mandibular ramus fracture model, on day 3 after fracturing, undifferentiated mesenchymal cells enveloped the bone surface around the fracture site. During this period, Bmp3, type I, II and X collagen was not detected in the mesenchymal cells. On day 7 after fracturing, Bmp3 was strongly detected in condensed fibroblast-like mesenchymal cells that expressing type I collagen and proliferating/mature hypertrophic chondrocytes that expressed type II and X collagen. On day 14 after fracturing, Bmp3 was observed in both type II collagen-expressing chondrocytes and type X collagen-expressing hypertrophic chondrocytes. In external callus, by day 3 after fracturing, an external callus had formed. Bmp3 could be detected in osteoblasts, while type I collagen was also expressed at the same site. On day 7 after fracturing, Bmp3 was expressed by active osteoblasts and osteocyte-like cells, at the same time, Bmp3-positive osteoblasts strongly expressed type I collagen mRNA.

[Discussion]

The present study, for first the time, has clearly localized Bmp3 mRNA in mandibular condylar cartilage, femoral growth plate cartilage, and the femoral articular cartilage, each of which showed well-aligned chondrocyte layers. Our findings suggest that Bmp3 expression in the special layer of typical articular cartilages may be regulated by mechanical stress stimulation. We also found that Bmp3 was expressed in the external callus during fracture healing. The function of Bmp3 should be investigated in further experiments.
論文審査の結果の要旨

軟骨は、個体発生的に成長板軟骨や関節軟骨のような一次軟骨と下顎頭軟骨のような二次軟骨に分類され、機械的負荷を受けているものとほとんど機械的負荷を受けていないものが存在する。一方、BMP3は脱灰骨中に多く存在することが報告されているが、その役割に関しては不明な点が多い。本研究は、機械的負荷を受けている下顎頭軟骨、大腿骨関節軟骨、下顎枝骨析治癒過程に出現する軟骨およびほとんど機械的負荷を受けていない大腿骨骨端成長板軟骨におけるBmp3 mRNAの発現様相を明らかにするため、6週齢ラットを用い、これらの異なる4種類の軟骨に存在する軟骨細胞をI型、II型、X型コラーゲンを用いて分化段階を同定し、様々な分化段階の軟骨細胞におけるBmp3 mRNAの発現をin situハイブリダイゼーションにより詳細に検索したものである。

その結果、Bmp3は、大腿骨骨端成長板軟骨では肥大软骨細胞に限局して認められたのに対し、下顎頭軟骨、大腿骨関節軟骨、下顎枝骨析治癒過程に出現する軟骨では、増殖軟骨細胞、成熟軟骨細胞、肥大軟骨細胞に発現した。以上の結果、BMP3は軟骨において機械的刺激の調節に関わっている可能性が示唆された。また、骨折後早期において、骨折部付近の骨膜層に存在する骨芽細胞にもBmp3は強く発現した。本研究の結果、BMP3は下顎頭軟骨の成長、維持に関わる下顎頭軟骨細胞の機械的刺激に応答する因子である可能性が示唆されたと考える。

よって、本研究は臨床と密接に関係した基礎的研究であることが高評価され、本申請論文は博士（歯学）の学位論文に値するものと認めた。