MMP-13 appears to be one of the most important MMPs in cartilage remodeling and mineralization, because it exhibits a substrate preference for the cartilage-specific type II collagen. The condylar process is constructed by rapid accumulation of hypertrophic chondrocytes during development, but its mechanism is still unclear. To investigate the role of MMP-13 in developing condylar cartilage, we immunohistochemically examined the localization of MMP-13 in the endochondral ossification of the mandibular condyle and tibiae of newborn mice. In the tibiae, the MMP-13 expression was detected only in the deepest layer of the terminal hypertrophic chondrocytes through every examined stage (day 1 to day 10 after birth). On the other hand, in the condylar cartilage at days 1 and 5, MMP-13 was expressed throughout the proliferating and the hypertrophic chondrocytes, and at day 10, MMP-13 was mainly localized in the deepest edge of the hypertrophic layer. A zymographical study showed that the activity of MMP-13 in the condyle was observed at day 1, earlier than in the tibia, and increased until day 7. The time-dependent and cell-specific expression of MMP-13 and its enzymatic property suggest that in the mandibular condylar cartilage, MMP-13 plays a role in making the space for cell enlargement by degradation of the cartilage matrix and in onset of mineralization during the early stage of development.

**Key words**: MMP-13, condyle, immunohistochemistry, gelatin zymography, endochondral ossification

**Introduction**

Mandibular condyle is produced by the endochondral ossification like that of long bone, however, its developing pattern is different from that of long bone. Mandibular condylar cartilage is often classified as a secondary cartilage, which develops from cells that have already differentiated into periosteum-like cells\(^1,2\) rather than from undifferentiated mesenchymal cells, and which is different from the primary cartilaginous skeleton in terms of its rapid progress from progenitor cells to hypertrophic chondrocytes. The temporal condylar process is constructed by rapid accumulation of these hypertrophic chondrocytes during the early stage of condylar development\(^2,5\). During development, there is an extensive growth and remodeling of tissues that involves the proteolysis of extracellular matrix\(^6\). For the rapid accumulation of hypertrophic chondrocytes, effective degradation of extracellular matrix, which is mainly composed of type II and type X collagen and proteoglycan, must be required. It has been shown that matrix metalloproteinases (MMPs) are associated with chondrocytes hypertrophy\(^7\) and carti-
MMPs comprise a family of enzymes that regulate cell differentiation and tissue remodeling through degradation and modification of the extracellular matrix. MMPs are produced by most cell types and their expression is transcriptionally regulated by growth factors and cytokines. Furthermore, the proteolytic activities of MMPs are regulated by the activation from their precursor form by a cascade involving other MMPs as well as the tissue inhibitors of metalloproteinases (TIMPs).

MMP-13 seems to be one of the most important MMPs in cartilage remodeling and mineralization, because it exhibits substrate preference for cartilage-specific type II collagen, and it can also degrade type X collagen and cartilage aggregan. In addition, Cbfa1, a transcription factor that induces the differentiation of chondrocytes activates the transcription of MMP-13. In mice lacking Cbfa1, chondrocyte differentiation is blocked and remained at the stage of prehypertrophic chondrocytes, indicating that MMP-13 is important for cartilage formation.

MMP-13 is normally expressed in hypertrophic chondrocytes, periosteal cells, and osteoblasts. However, the role of MMP-13 in chondrogenesis of the mandibular condylar cartilage is still unknown. The purpose of this study is to clarify the role of MMP-13 during the growth and development of condylar cartilage. To elucidate the function of MMP-13, we investigated the immunohistochemical localization of MMP-13 in the developing mouse mandibular condylar cartilage in vivo and the zymographical study of MMPs.

Materials and Methods

Animals and Tissue Preparation

The animal experimental protocol used in this study was approved by the Institutional Review Board of the Tokyo Medical and Dental University and was performed in accordance with the guidelines of this review board. Institute of Cancer Research (ICR) mice were used for this study of the endochondral bone formation in the tibia and condyle. The mice (day 1, day 3, day 10) were sacrificed by cervical dislocation under anaesthesia, and the tibiae and the mandibular condyles were resected using scissors. All the specimens were fixed immediately by immersion in 4% paraformaldehyde in 0.1 M phosphate buffered saline, pH 7.4 (PBS) for 1 day at 4 °C and decalcified with 10% EDTA for 3 days at 4 °C. All tissues were processed and embedded in paraffin. Sections (5 µm) were cut longitudinally to the tibia and parallel to the long axis of the condylar process of the mandible. Some of the sections were stained with 0.1% toluidine blue (0.1M phosphate buffer, pH 7.4) for routine morphological evaluation.

Immunohistochemistry

To evaluate the localization of the MMP-13 protein, immunohistochemical staining was performed by the CSA (Catalyzed Signal Amplification) system (Dako, Carpinteria, CA) according to the manufacturer’s instructions. The methods used in this system were based on the avidin-biotin and the peroxidase methodologies. Briefly, the specimens were first incubated with 3% hydrogen peroxide for five minutes to quench the endogenous peroxidase activity. The specimens were then incubated for five minutes with a blocking reagent (0.25% casein in PBS) to suppress nonspecific binding of the subsequent reagents, followed by incubation with an appropriately characterized and diluted primary antibody or negative control reagent. This was followed by sequential 15-minute incubations with the biotinylated link antibody, streptavidin-biotin-peroxidase complex, amplification reagent, and streptavidin-peroxidase. Staining was completed by a 5-minute incubation with 3, 3’-diaminobenzidine tetrahydrochloride (DAB) which results in a brown-colored precipitate at the antigen site. Each step in the staining procedure was performed at room temperature. The primary antibody used in this study was the anti-MMP-13 antibody (CHEMICON International, Inc. Temecula, CA). For the negative controls, PBS or normal goat IgG was used instead of the primary antibodies. The sections were counterstained with methyl-green.

Gelatin Zymography

The tibiae and condyles (day 1, day 3, day 7, day 10, day 14) were homogenized in extraction buffer (20 mM Tris, pH 8.0, 0.5 mM CaCl₂, 0.5 % NP-40) and the protein concentration of the supernatant after centrifugation (10,000 x g, 10 min) was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Aliquots of each sample (50 µg/10 µl) were mixed with 20 µl of the non-reducing sample buffer consisting of 500 mM Tris-HCl (pH 6.8), 25% glycerol, 10% SDS, and 1% bromophenol blue and applied using a Gelatin Zymography Kit (Yagai, Yamagata, Japan) according to the manufacturer’s instructions. After electrophoresis, the gel was incubated in the renatura-
tion buffer at room temperature for 1hr then in the developing buffer at 37 °C overnight as indicated in the instruction manual. The gel was rinsed and stained with Coomassie blue. After destaining, a light translucent band over a blue background was detected for gelatinase activity.

Results

**Histological changes in developing tibia and condyle**

To investigate the immunolocalization of MMP-13, we used the tibial and condylar cartilage from three developing phases (day 1, day 5, and day 10 after birth) in the growth process of mice. In the tibia of day 1 mice, the epiphyseal plate exhibited cellular morphology characteristics of the epiphyseal plate cartilage with four successively layered zones of chondrocytes with distinct appearances. From the epiphysis to the diaphysis, these were the resting zone, the proliferating zone, the hypertrophic zone and the trabecular bone. The cartilage-bone transitional plane is directed vertically to the tibial shaft axis, and vascular invasion of cartilage at the cartilage-bone transitional plane is observed (Fig. 1A).

The changes in the tibiae between day 1 and day 10 of age are characterized by expansion of its width.

![Histological changes in developing tibia and condyle](image-url)
Furthermore, as to the shape of the epiphysis, expansion toward the top was observed in the tibia of day 10 mice caused by the occurrence of the secondary ossification center (Fig. 1C).

In the condyle of day 1 mice, the cartilage-bone transitional plane is oriented vertically to the condylar developing axis and the proliferating and hypertrophic zones are very thick (Fig. 1D). On day 5, the cartilage-bone transitional plane has leaned in the upper direction. The proliferating and hypertrophic zones are still thick, but the hypertrophic zone becomes thin compared to day 1 (Fig. 1E). On day 10, the cartilage-bone transitional plane has advanced toward the articular surface. Not only the proliferating zone but also the hypertrophic zone become narrower compared to day 1 and day 5, but are still present. Most of the chondrocytes have been replaced by osseous tissue (Fig. 1F).

**Immunohistochemistry of MMP-13 protein**

In the tibiae, the MMP-13 expression was strongly detected in the deepest layer of the terminal hypertrophic chondrocytes and the expression was weak in the proliferating chondrocytes through every stage studied (Fig. 2A-C). Negative controls for MMP-13 did not show any specific expression in the tibia. r: resting cell zone; pr: proliferative cell zone; hc: hypertrophic cell zone; b: bone. Bar= 250 µm

![Figure 2](image-url)
condylar cartilage on day 5, MMP-13 is strongly expressed throughout the proliferating chondrocytes and the hypertrophic chondrocytes (Fig. 3B). In the condylar cartilage on day 10, the immunolocalization of MMP-13 was mainly detected in the deepest layer of the terminal hypertrophic chondrocytes that have reduced its thickness and was less observed in the proliferating chondrocytes (Fig. 3C). Negative controls for MMP-13 (D-F) did not show any specific expression (Fig. 3D-F).

In the deepest layer of the terminal hypertrophic chondrocytes, the MMP-13 expression was detected in the hypertrophic chondrocytes adjacent to the new bone and in the osteoblastic cells of the trabecular bone. The localization of MMP-13 was similar in the tibia and in the condylar cartilage (Fig. 4A, B). Due to the resolution of the immunohistochemical sections, it could not be identified whether the osteoclasts exhibit the MMP-13 expression or not.

Gelatin Zymography of MMPs in the tibia and the condylar cartilage

To evaluate the activity of the MMPs in the tibia and condyle, we carried out gelatin zymography. The level of pro MMP-9 (92 kDa) and active MMP-9 (~80 kDa) in the tibia, especially the active form of MMP-9, was low on day 1 and increased with the animal's age. The level of MMP-9 in the mandibular condyle from day 1 to day 7 was higher than in the tibia. The levels of pro MMP-2
(72 kDa) and active MMP-2 (~64 kDa) in both tissues were almost constant during the ranges of age examined (Fig. 5). Bands below the active MMP-2 band might be due to MMP-13 since it has potent gelatinase activity, and the activated MMP-13 would be expected below 60 kDa. MMP-13 activity in tibia was first detected on day 7, and seemed to increase gradually until day 14. However, the activity of MMP-13 in the condyle appeared on day 1, earlier than in the tibia, and increased until day 7 and did not change again until day 14 (Fig. 5).

**Discussion**

The hypothesis that cartilage-derived proteinases were required for initiation of mineralization was proposed 25 years ago\(^2\). Recently, it has been shown that MMPs play important roles in the degradation and calcification of the cartilage matrix and vascular invasion into the cartilage. MMPs comprise a family of extracellular matrix (ECM)-degrading enzymes that shares common functional domains and activation mechanisms. These are Ca\(^{2+}\) - and Zn\(^{2+}\)-dependent endopeptidases that are active at neutral pH. There are now more than 20 members of the MMP family. The
expression of MMPs during the development of endochondral ossification has rarely been reported\(^{31,32}\). Some reports concerning the expression of MMPs in condylar cartilage were carried out at different stages of embryogenesis.

In this study, we demonstrated that the expression of MMP-13 is associated with the changes in the rapid extension of the condylar process in the young mouse. The MMP-13 protein was observed in the proliferating chondrocytes, the hypertrophic chondrocytes and the cells at the resorption front of the growing mandibular condyle (Fig. 3 A-C). We revealed the localization of MMP-13 in the layer of condylar cartilage and the time specific appearance during the developmental stage precisely, consisting with the previous report\(^{29}\).

Matrix degradation by proteinases may be essential for chondrocyte enlargement and rapid proliferation in condylar cartilage, which occurs at a stage before the onset of mineralization. In this study, we revealed the differences in the expression pattern of MMP-13 between the developing mouse condylar cartilage and tibia. The data revealed that MMP-13 was evenly expressed not only in the hypertrophic chondrocytes but also in the proliferating chondrocytes during the early developing mouse condylar cartilage in vivo. The strong positive MMP-13 reaction obtained in the proliferating chondrocytes supports the speculation that MMP-13 plays an important role in the enlargement of chondrocytes in the developing condyle.

In the both tibia and condylar cartilage on day 10, a strong positive reaction of MMP-13 was observed in the hypertrophic chondrocytes and osteoblast-like cells in the deepest edge of the hypertrophic layer where the cartilage matrix was replaced by bone matrix (Fig 4A, B). This observation is comparable with a previous report showing MMP-13 mRNA and the protein expression in hypertrophic chondrocytes and osteoblasts during development\(^{30}\). A similar observation of the MMP-13 expression in the mouse fracture callus was also reported\(^{31}\). In addition, it has been known that cells expressing MMP-13 were observed very close to the TRAP-positive cells, but these two cells did not overlap\(^{31,32}\). Therefore, it is indicated that cells expressing MMP-13 cooperate with osteoclasts for matrix degradation and resorption and that MMP-13 is important for the matrix degradation required for the initiation of the mineralization and migration of the osteoblastic cells.

In the tibia on day 10 mice, immunoreaction of MMP-13 in the boundaries between proliferating chondrocytes and hypertrophic chondrocytes was very low (Fig. 2C). Low levels of MMP-13 expression in this region might reflect that matrix turnover of this region at this stage is not so high as 1-day- or 5-day-mice.

The results of the gelatin zymography showed that the activity of MMP-13 in the condyle appeared on day 1, earlier than in the tibia, and increased until day 7. The MMP-13 activity in tibia, however, was first detected at day 7. The level of MMP-13 activity in the condyle was higher than in the tibia until day 10 (Fig. 5). These findings are consistent with the results of the present immunohistochemistry and coincide with the previous report in newborn mice in which the message of MMP-13 gradually increased from day 2 to day 14\(^{29}\). Because mandibular condylar cartilage has a larger number of hypertrophic chondrocytes than the tibia in the development stage observed in this study, the higher activity and earlier appearance of MMP-13 in the condyle in the gelatin zymography further supports the speculation that MMP-13 plays an important role in enlargement of the chondrocytes.

The activity of MMP-9 in the tibia, especially the active form of MMP-9, was low on day 1 and increased with the animal’s age. On the contrary, the level of MMP-9 in the mandibular condyle from day 1 to day 7 was higher than in the tibia. Recently, it was reported that the expression level of the mRNA of MMP-9 in the tibia increased during a similar period to the present study\(^{29}\). The zymographical observations for MMP-9 in the tibia and condyle indicated that the role for this proteinase is to remove the denatured collagen fragments by the gelatinolytic activity of MMP-13\(^{11}\).

The levels of pro MMP-2 and MMP-2 in both tissues were almost constant during the examined ranges of age (Fig. 5). This is consistent with the speculation that MMP-2 has a housekeeping function of the normal turnover of cartilage\(^{11,33,34}\). Gepstein et al. also reported that the expression level of mRNA in MMP-2 of the tibial cartilage was not altered in the newborn mice from day 2 to day 14 by RT-PCR\(^{29}\). Because it has been suggested that MMP-13 is activated by MMP-2 during chondrocyte maturation\(^{35}\), the combination of both proteinases may be required to prepare the cartilage matrix for subsequent calcification both in the tibia and condyle.

Of the MMP family, MMP-13 seems to especially contribute to the endochondral ossification, cell enlargement during chondrocyte hypertrophy and matrix calcification at the chondro-osseous region. It has been reported that matrix vesicle-associated MMP13 activates chondrocytes-produced latent TGF-β
and may be required for osteoblastic differentiation. Inhibition of the matrix degradation by RS 102,481, a carboxylate inhibitor of MMP-13, arrested the type II collagen degradation and had a strong inhibitory effect on the chondrocyte differentiation in vitro. This inhibition accompanied by the suppression of Cbfa1 mRNA expression and suppression of the Ca incorporation into the cell layers of the cultured hypertrophic chondrocytes. Cbfa1, an MMP-13 transcription factor, is an important factor for chondrocyte maturation. Wu et al. indicated that the Cbfa1/MMP-13 pathway plays an important role in chondrocyte differentiation as well as in the matrix turnover. Therefore, it is indicated that the activity of MMP-13, as shown in this study, is required for chondrocyte differentiation in the growth plate of the mandibular condyle.

Together with the previous reports and the present observations, it is indicated that during the growth and development of the condylar cartilage, MMP-13 produced by proliferating cartilage functions to make space for cell enlargement, and that MMP-13 in the hypertrophic chondrocytes functions for matrix degradation required for the initiation of the mineralization. The time-dependent and cell-specific expression of MMP-13 and its enzymatic property accomplish the role to make the mandibular chondylar cartilage during the early stage of development.

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