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SCIENTIFIC SECTION

Local administration of IGF-I stimulates the growth of mandibular condyle in mature rats

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Aim: To evaluate the effects of local injection of insulin-like growth factor I (IGF-I) on the growth of mandibular condyle in mature rats.

Materials and methods: Sixteen 15 week-old male rats were used in this study. In the experimental group, IGF-I at a concentration of 50 μg/ml was injected into articular capsules of the condyle, while rats in the control group were injected with equal volume of physiological saline. These injections were performed three times at 7 day intervals, and all of the rats were killed on the 7th day after the last injection. Tetracycline and calcein were used for vital staining. After death, the condyles were extracted and undecalcified ground sections were prepared for histological and histomorphometric observations. The thickness of the cartilaginous layer of the condyle, the percentage of bone area in the subchondral cancellous bone layer and the amount of endochondral bone growth in the condyle were measured. The significance of the difference in these measurements between IGF-I and control group was evaluated.

Results: An increase in the thickness of the cartilaginous layer, and a decrease in the percentage of bone area in the subchondral cancellous bone layer was recognized in IGF-I treated condyle. The amount of endochondral bone growth in the experimental group was greater than that in the control group.

Conclusion: The local injection of IGF-I into mature condyle seemed to reactivate the process of endochondral bone formation and induced actual bone growth in mature condyle.

Key words: Endochondral bone formation, insulin-like growth factor-I, local administration, mandibular condyle, rat

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Introduction

Controlling the growth of the maxillofacial skeleton is an important theme for research in the field of clinical orthodontics. In studies on maxillofacial growth following the administration of growth hormone to patients with pituitary dwarfism, the amount of growth in the mandible is greater than that in other parts of the maxillofacial skeleton.[1] Remarkable growth of the mandible has also been reported in patients who underwent treatment with growth hormone for poor general growth caused by some other congenital diseases or bone marrow transplant.[2] These results suggest that the effects of growth hormone differ at various sites in the maxillofacial skeleton and, especially, growth of mandible seems to be particularly activated by exogenous growth hormone. Therefore, it may be possible to selectively promote growth at a specific site of the maxillofacial skeleton by adjusting the timing and method of administration by using these differences in the response of the maxillofacial skeleton to hormones and/or growth factors.

IGF-I is synthesized and secreted in the liver and other organs following stimulation by growth hormone. It accelerates growth, differentiation and substrate synthesis activity in osteoblasts and chondroblasts.[3–9] Furthermore, IGF-I has been reported to play important roles in the growth of long bone as well as the growth of mandibular condyle.[10,11] Itoh *et al.* noted that the histological changes induced in the mandibular condyle after the local administration of IGF-I differed between 3 and 12 week-old rats.[12] Interestingly, the histological structure of mature condyle in 12 week-old rats changed to resemble that in a younger growth stage following the local administration of IGF-I. However, it is unclear whether these histological changes are temporary or are accompanied by actual growth of the condyle. Accordingly, in the present study, we sought to confirm whether or not condylar growth is activated by local administration of IGF-I by measuring the changes of

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thickness of the cartilaginous layer and the amount of endochondral bone formation.

The purpose of this study was to identify the effects of the local administration of IGF-I on the actual growth of mandibular condyle by using the technique of vital staining.

Materials and methods

Experimental animals

Sixteen 15-week-old male Sprague–Dawley rats (Sankyo Lab Service Co., Inc., Tokyo, Japan) were used in this study. They were equally divided into an experimental group and a control group. All of the rats were weighed once a week during the experimental period.

All experimental procedures adopted in this study were approved by Animal Care and Use Committee and the experiment has been properly carried out under the control of the Guideline for Animal Experimentation in Tokyo Medical and Dental University (Approval No.40296).

Method of local administration to the condyle

Local administration to the condyle was performed by the method described by Itoh *et al*.[12] Initially, each rat was anesthetized intraperitoneally with sodium pentobarbital. A small incision was made in the skin between the eye and ear and, after the occlusal muscles were moved aside, the location of the articular capsules of the temporomandibular joint was identified. Next, 0.02 ml of a solution of insulin-like growth factor I (rhIGF-I, R&D Systems, Minneapolis, MN) at a concentration of 50 μg/ml was injected into the articular capsules on both sides in the rats in the experimental group using a tuberculin syringe (27 gauge needle, 0.75 inch syringe, Terumo, Tokyo, Japan). After these injections were complete, the incision in the skin was closed by suturing. The rats in the control group were injected similarly with an equivalent volume of physiological saline. These injections were performed three times at 7 day intervals and all of the rats were killed on the 7th day after the final administration. For vital staining, all of the rats were given a solution of tetracycline (8 mg/kg) just before the administration of IGF-I or physiological saline. Similarly, a solution of calcein (5 mg/kg) was injected 5 hours before death.

Preparation of sections

After death, the mandibular condyles on both sides were dissected out and fixed with Karnovsky's fixative solution (pH 7.2). Left condyles were dehydrated through an ethanol series and embedded in acrylic resin (LR white resin, London Resin Co. Ltd, UK). In order to obtain a standardized mid-sagittal plane of section in each condyle, the method described by Suzuki was adopted.[13] Namely, before embedding in acrylic resin, three reference points of condylar head (anterior edge, posterior edge and midpoint of anterior and posterior edge on the uppermost articular surface in frontal dimension) were marked directly on each condyle. In preparing undecalcified ground sections, the orientation of the section was adjusted so that these three reference points should be lined up on the same plane. The thickness of the ground section was about 50 μm. After fluorescent labeling was observed, undecalcified ground sections were stained with a 1.0% aqueous solution of Azure A (pH 5.4) and counterstained with a 1.0% aqueous solution of toluidine blue O (pH 6.8) for histological observation and histomorphometric analysis. Some of the right condyles were decalcified and embedded in water-soluble resin, and serial decalcified sections were prepared to assist in the histological observations.

Histomorphometric measurement

Thickness of the cartilaginous layer.
In light microscopic observations, *x*- and *y*-axes were set up on undecalcified ground sections for histomorphometric measurement. The *x*-axis was defined as the line from the anterior edge between cartilage and bone (point A) to the posterior edge between cartilage and bone (point B) on the mid-sagittal plane of the condyle. The *y*-axis was defined as the line perpendicular to the *x*-axis that passed through point C, which fell at two-thirds of the distance from points A and B. The thickness of the cartilaginous layer of the condyle was measured as the distance from the articular surface to the bottom of the cartilaginous layer along the *y*-axis in each ground section using a micrometer under a light microscope (Figure 1A).

Percentage of bone area in the cancellous bone layer.
To evaluate bone-remodeling activity in the subchondral cancellous bone layer, the ratio of bone area to total tissue (percentage of the bone area) within a 0.5×0.5 mm square was calculated under light microscope using image-analysis software (Mac Scope, Mitani Corporation, Tokyo, Japan). The square was positioned so that its vertical edge was parallel to the *y*-axis of the ground section, and its upper horizontal edge was placed on the upper edge of the cartilage lacuna, which initially opened to the bone marrow (Figure 1A).

Amount of endochondral bone growth in condyle.
Endochondral bone growth in the condyle during the experimental period was estimated by measuring the

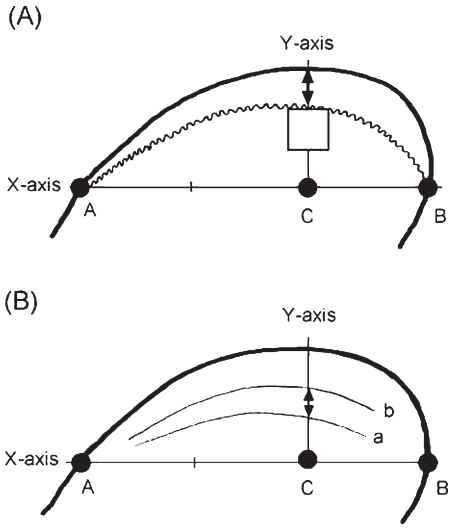

Figure 1 Diagram of the reference axes on a mid-sagittal section from mandibular condyle and parameters in histomorphometric measurements. (A) Point A, the anterior edge between the cartilage and bone; Point B, the posterior edge between the cartilage and bone; and Point C, a reference point which falls two-thirds of the distance between points A and B. *x*-axis, line AB; *y*-axis, a line perpendicular to the *x*-axis that passes through point C. The vertical arrow indicates the thickness of the cartilaginous layer of the condyle. The square shows the measured area of bone in the subchondral cancellous bone layer of the condyle. Line a, fluorescent label of tetracycline; Line b, fluorescent label of calcein. Vertical arrow indicates the distance between the two fluorescent labels

distance between the calcein and tetracycline labels. The amount of endochondral bone growth in condyle was measured as the distance between the calcein and tetracycline labels along the *y*-axis (Figure 1B). To evaluate the significance of the difference in these measurements between the IGF-I and control groups, Student's *t*-test was performed using Stat View (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

Changes in body weight during the experimental period

A slight increase in body weight was recognized during the experimental period in both experimental and control groups. However, no significant difference in body weight was found between the experimental and control groups.

Histological observation and measurement of histomorphometric parameters

Histological observation.
Figure 2 shows mid-sagittal sections of the condyle in the control and experimental groups. In the control group, the cartilaginous layer was thin (Figure 2A). On the other hand, in the experimental group, a significant increase was observed in the thickness of the cartilaginous layer and especially in the thickness of the hypertrophic chondrocyte layer (Figure 2B).

Thickness of the cartilaginous layer.
The thickness of the cartilaginous layer in the control and experimental groups is compared in Figure 3. The cartilaginous layer in the experimental group (452 ± 30 μm) was significantly thicker than that in the control group (295 ± 40 μm; $p < 0.05$).

Percentage of bone area in the subchondral cancellous bone layer.
Figure 4 shows the percentage of bone area in the subchondral cancellous bone layer in both groups. The percentage of bone area in the subchondral cancellous bone layer in the experimental group ($25.7 \pm 1.7\%$) was significantly less than that in the control group ($37.6 \pm 2.0\%$; $p < 0.05$).

Amount of endochondral bone growth.
Figure 5A shows a fluorescent microscopic image of the condyle in the control group and Figure 5B shows that in the experimental group. The lines of tetracycline and calcein can be recognized. The distance between the two labeling lines reflects the amount of endochondral bone growth in the condyle during the experimental period. Figure 5C shows the amount of endochondral bone growth in the condyle: the amount of endochondral bone growth in the experimental group (375 ± 39 μm) was greater than that in the control group (310 ± 41 μm; $p < 0.05$).

Discussion

Experimental design

Itoh *et al.* reported that the local administration of IGF-I to the condyle had a greater effect in mature rats than in younger rats in the rapid growing period.[12] Accordingly, the rats in the present study were selected to be 15 weeks old. They were sexually mature, but not so old that they showed a decreased biological response.

In order to ensure lasting effects of IGF-I on condylar growth during the experimental period, injections of IGF-I were performed three times at 7 days intervals. The histological changes observed at the 7th day after the final injection of IGF-I in the present study were similar to those observed at 3 days after IGF-I administration in the study by Itoh *et al.* In this latter study the concentration of IGF-I was 20 μg/ml, administered in a single dose.[12] These histological changes included an increased

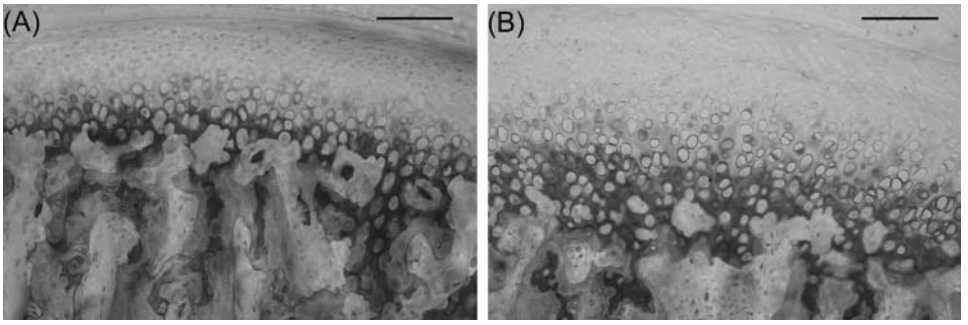

Figure 2 Light microscopic observations in the condyle. (A) Control group. (B) Experimental group. An increase in the thickness of the cartilaginous layer was observed in the experimental group. Bar = 100 μm

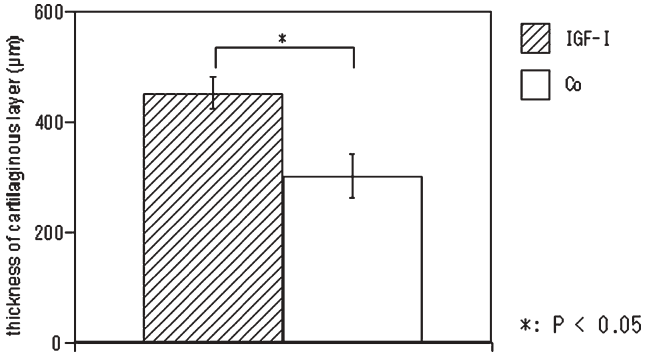

Figure 3 Thickness of the cartilaginous layer. The thickness of the cartilaginous layer was increased in the experimental group. $p < 0.05$ ($n = 8$ for each group)

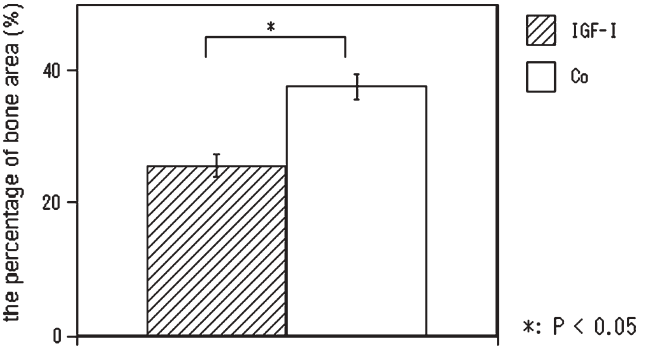

Figure 4 Percentage of bone area in the subchondral cancellous bone layer. The percentage of bone area in the subchondral cancellous bone layer in the experimental group was significantly less than that in the control group. $p < 0.05$ ($n = 8$ for each group)

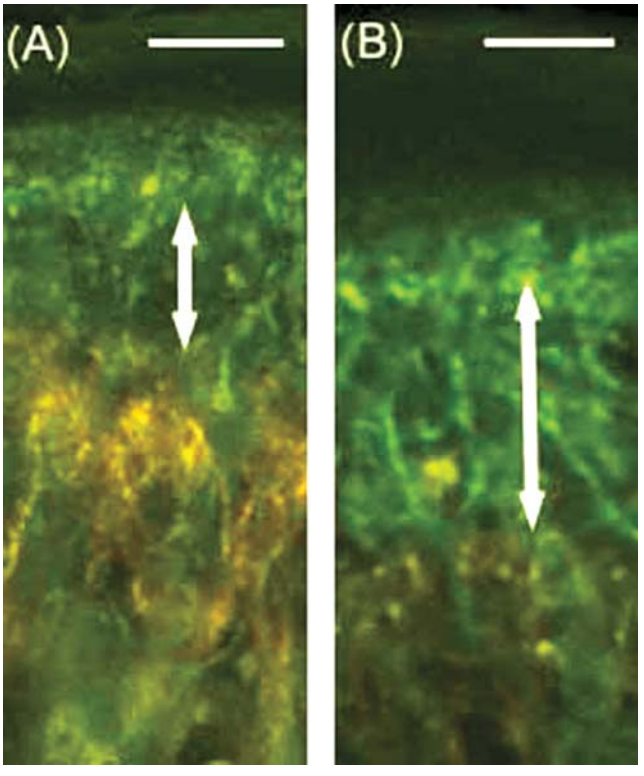

Figure 5a & b

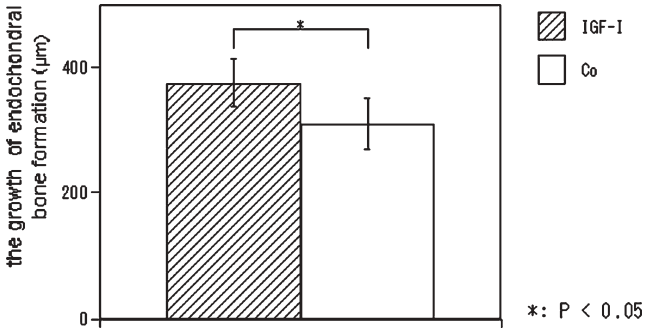

Figure 5 Amount of endochondral bone growth. Fluorescent microscopic observation of condyle in the control group (5A) and experimental group (5B). The distance between the two labeling lines, tetracycline and calcein, was measured as endochondral bone growth during the experimental period (vertical arrow). The amount of endochondral bone growth in the experimental group was significantly greater than that in the control group. Bar = 250 μm. $p < 0.05$ ($n = 8$ for each group)

thickness of the cartilage layer and a reduced bone area in the subchondral cancellous bone layer. In the study by Itoh, these histological changes returned to normal at 5 days after administration. In the present study, histological changes in the condyle persisted for 7 days after IGF-I administration, which suggested that the effects

of IGF-I on condylar tissue continued during the experimental period.

Histomorphometric measurements

The labeling line produced by vital staining in condylar cartilage reveals sites at which the calcification of cartilage had begun. In the present study, the distance between the two labeling lines of tetracycline and calcein was used to indicate the amount of cartilage replaced by bone between separate administrations of fluorescent pigment; i.e. it revealed the amount of endochondral bone growth of the condyle during the experimental period. The results of our study indicated IGF-I not only changes the histological structure of the condyle, but also activates endochondral bone formation and induces actual condylar growth. In this experiment, the local administration of IGF-I increased both the thickness of the cartilaginous layer and endochondral bone growth. When the labeling line of tetracycline administered at the start of the experiment was used as a common baseline in both the experimental and control groups, the growth of the condyle in the experimental group was calculated to be due to an increase in the thickness of the cartilage layer together with an increase in endochondral bone growth.

The effects of IGF-I on mandibular condyle and the growth plate of long bone have been analyzed using both *in vivo* and *in vitro* experimental systems, and IGF-I is known to contribute to cartilage growth by promoting the proliferation and differentiation of chondroblasts.[14] In rats whose pituitary gland has been experimentally removed, blood IGF-I levels decreased and the growth plate cartilage became thinner. The local administration of IGF-I to the growth plates of such rats has been reported to increase the thickness of growth plate cartilage, and such histological changes are concluded to be the result of a direct effect of IGF-I on chondrogenic cells.[7–9] Since the production of endogenous IGF-I in the mature rats is estimated to be less than that in a younger growth stage, increase in the thickness of the cartilage layer in this study is also considered to be a direct effect of IGF-I on chondrogenic cells in condyle.

Spencer *et al.* reported that bone tissue was increased in the subchondral cancellous bone layer in a study in which IGF-I was administered locally to the growth plate of non-hypophysectomized rats.[15] Since local administration in their study was performed via the blood using a cannula, it is believed that osteogenic cells in the subchondral cancellous bone area were directly affected by IGF-I from the bone marrow side via the blood. On the other hand, in our experiment, IGF-I may have had less of an effect on cells in the subchondral cancellous bone layer, since it was injected into the joint capsule.

The bone area in the subchondral cancellous bone layer in the pre-mature condyle in which endochondral bone formation is actively progressing has been reported to be small, but increases with age.[13] Thus, the decrease in bone area in the subchondral cancellous bone layer in the present study was thought to be caused by the reactivation of bone remodeling in this area. Although the factors that promoted the activity of bone remodeling in the subchondral bone layer in the present study are not clear, the proliferation of chondroblasts and the accumulation of hypertrophic chondrocytes induced by the local administration of IGF-I may have an indirect effect on bone remodeling in subchondral tissue.

Further growth of mature condyle

In contrast to the growth plate of long bone, which is completely replaced by bone at the termination of general growth, condylar cartilage continues to remain articular cartilage even after general growth is complete. Patients with acromegaly show characteristic mandibular protrusion. This pathological condition is known to be caused by the excessive secretion of growth hormone mainly due to pituitary adenoma, and the IGF-I level in the blood of these patients has been reported to be significantly elevated.[16,17] In morphological analyses of the facial skeleton of such patients, the mandibular body is enlarged and the mandibular ramus also appears to be remarkably long.[18,19] These findings regarding the excessive growth of the mandible, especially the increased length of the mandibular ramus, may be associated with the reactivation of condylar growth. Thus, in the case of acromegaly, even though general growth has either slowed or terminated, the condyle is still activated due to the effects of excessive growth hormone and/or IGF-I, which cause growth to resume. The present finding, where the growth of mature condyle was reactivated by the local administration of IGF-I, may serve as a model for explaining the mechanism of skeletal mandibular protrusion in acromegaly. Moreover, the clinical application of these findings may make it possible to maintain condylar growth beyond termination of general growth.

Conclusion

The effects of local administration of IGF-I on the condyle were analyzed in the 15 week-old mature rats using a vital staining technique. Histological changes, such as an increase in the thickness of the cartilaginous layer and a decrease in bone area in the subchondral cancellous bone layer, were observed in the IGF-I-treated group. In addition, the measurement of labeling lines produced by vital staining revealed that the amount of endochondral bone growth in the experimental group

was greater than that in the control group. These results indicate that the local injection of IGF-I induced not only temporary histological changes, but also actual skeletal growth of the condyle.

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Authors and Contributors

SS was responsible for study design, handling laboratory experiments, data collection, data analysis and interpretation, drafting, critical revision and final approval of the article. KI was responsible for handling laboratory experiments, technical support, data collection and analysis, and final approval of the article. KO was responsible for obtaining funding, data interpretation, critical revision and final approval of the article. SS is the guarantor.

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