Possible alternative treatment for mandibular asymmetry by local unilateral IGF-1 injection into the mandibular condylar cavity: Experimental study in mice

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Introduction: The purpose of this study was to investigate whether a local unilateral IGF-1 injection into the mandibular condylar cavity can induce unilateral endochondral mandibular growth without any systemic adverse effects. **Methods:** Seventy-five 3-week-old male Jcl:ICR mice were used in this study. The mice were divided into 2 groups: control group (n = 22) and IGF-1 group (n = 53). In the IGF-1 group, human IGF-1 was injected into the right mandibular condylar cavity, and phosphate-buffered saline solution was injected into the left cavity, 3 times per week for 10 weeks. **Results:** There was no significant difference in body weight, serum human IGF-1 concentration, and soft tissue thickness of the cheeks including the masseter muscles between the 2 groups. Unilateral IGF-1 injection induced a lateral shift of the mandible to the contralateral side, and microcomputed tomogtraphy analysis showed that unilateral IGF-1 injection induced endochondral growth in the condyle. Col2, Ihh, and Runx2 were extensively upregulated by the local unilateral IGF-1 injection in real-time reverse transcription polymerase chain reaction analysis. Proliferation marker KI67, IGF-1 signaling molecule AKT1, and chondrogenic differentiation marker Col2 were strongly expressed in the IGF-1 injected condyle by immunohistochemistry. Vital labeling showed that the distance between the labels was increased in the IGF-1 injection group compared with that of the control group. **Conclusions:** The results verified in this study indicated that local unilateral IGF-1 injection into the mandibular condylar cavity successfully induced unilateral endochondral mandibular growth in mice without any systemic adverse effects. Thus, local unilateral IGF-1 injection into the mandibular condylar cavity could be a useful alternative for mandibular asymmetry therapy during the growth period. However, additional experimental and clinical studies will be necessary to prove the real effect of this new therapy. (Am J Orthod Dentofacial Orthop 2017;152:820-9)

It is well known that symmetry of the maxillofacial bone is essential for ideal occlusion.[1,2] Letzer and Kronman[1] reported that a comparative study of frontal cephalograms between ideal occlusion and malocclusion demonstrated a positive relationship between maxillofacial symmetry and ideal occlusion. Therefore, skeletal asymmetry, especially mandibular asymmetry, would result in malocclusion.

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Factors for mandibular asymmetry are divided into congenital and acquired factors.[3,4] Of them, acquired factors for mandibular asymmetry are categorized into 2 types: functional mandibular deviation[5] and difference in the amounts of condylar growth between the sides.[6,7] Functional mandibular deviation often transforms into skeletal mandibular asymmetry during growth.[5] Several studies on artificial lateral displacement of the mandible reported increases in the articular condyle and mandibular growth on the contralateral side using various experimental models.[8-10] Therefore, functional lateral displacement of the mandible during growth should be corrected as early as possible to promote favorable balanced growth and development of the mandible for obtaining facial symmetry.[5,11] Another acquired factor is the disproportionate development of the mandible by different endochondral ossifications in both sides.[3]

Various growth factors such as GH[12], FGF[13], BMP[14], and IGF-1[12-14] have been implicated in endochondral

ossification. In particular, IGF-1 has been reported to be involved in growth by regulating endochondral ossification.[13] IGF-1 regulates growth not only in limb bones, but also in the mandible.[14-16] Iikubo et al[17] developed a rat model of acromegaly by continuous subcutaneous infusion of human recombinant IGF-1 using implanted osmotic minipumps, and the rats hadd mandibular enlargement. In addition, Maor et al[18] reported an in-vitro stimulative effect of IGF-1 on mouse primary condylar chondrocyte growth. A receptor for IGF-1, IGF-1R in rat condylar cartilage was also reported.[19] An artificial mandibular functional shift resulted in the increase in the gene expressions of both IGF-1 and IGF-1R in the condylar cartilage of the contralateral side, and increased the mandibular condylar growth on the contralateral side.[20] Furthermore, Itoh et al[19] reported that the local injection of IGF-1 into the bilateral mandibular condylar cavities caused increased endochondral bone formation in the mandibular condyle, and the phenomenon was age-dependent in rats. Accordingly, these studies suggest a therapeutic effect of the local injection of growth factor on the condylar growth of the patient.

However, to the best of our knowledge, no study has investigated the possibility of correction of mandibular asymmetry via unilateral mandibular growth control with local unilateral IGF-1 injection. Therefore, we investigated in a mouse model whether the local unilateral injection of IGF-1 into the mandibular condylar cavity can induce unilateral endochondral mandibular growth at that side without systemic adverse effects.

MATERIAL AND METHODS

The experimental protocol in animals was approved by the institutional animal care and use committee of Tsurumi University, Yokohama, Japan (approval numbers. 25P054, 26P041, 27P001, and 28P028), and carried out in accordance with the guidelines for animal experimentation of the university.

Seventy-five male Jcl:ICR mice, 3 weeks old (CLEA Japan, Tokyo, Japan), were used in this study. The mice were housed under specific pathogen-free conditions and fed powdered chow and tap water ad libitum. They were divided into the following 2 groups: control group (n = 22) and IGF-1 group (n = 53). In the IGF-1 group, the mice received a 20-μl (20 μg/site) intra-articular injection (see procedure of local IGF-1 injection below) of human IGF-1 solution (Somazon; Astellas Pharma, Tokyo, Japan) into the right mandibular condylar cavity, and 20 μL of phosphate-buffereed saline solution (PBS) into the left side.

Local injection of IGF-1 into the mandibular condylar cavity was performed by the method described by Kameoka et al[21] under anesthesia with the intraperitoneal injection of medetomidine 0.3 mg per kilogram of body weight, midazolam 4.0 mg per kilogram of body weight, and butorphanol 0.5 mg per kilogram of body weight. Successful local injection into the mandibular condylar cavity was confirmed by hematoxylin dye injection using 11 mice. In the control group, 20 μL of intra-articular injections of saline solution were given on both sides 3 times per week for 10 weeks. The mice were killed by cervical dislocation at 10 weeks after the first injection. The soft tissues of their heads were scanned by x-ray microcomputed tomography (microCT) (MFZ; Hitachi, Tokyo, Japan). After the taking of these images of soft tissues, the mandibular condyles were excised out and rapidly immersed in liquid nitrogen. The frozen tissues were then embedded in precooled optimal cutting temperature compound (Sakura Finetek, Torrance, Calif) and soaked in liquid nitrogen until the optimal cutting temperature compound was completely frozen. The frozen mandibular specimens in the optimal cutting temperature compound were scanned by microCT, and the images were reconstituted using cone-beam CT express software (TRI/3D Bone; RATOC, Tokyo, Japan). After reconstitution and obtaining the DICOM files, 3-dimensional volume rendering was performed using OsiriX 64 bit (Pixmeo, Bernex, Switzerland). The lengths of the mandibles were measured in millimeters from the mesial contour of the mandibular first molar to the midpoint of the mandibular condyle (Fig 1, *A-C*), and these were measured using multiplane reformation view in OsiriX 64 bit.

The soft tissues thicknesses (in millimeters) of the cheeks including the masseter muscles were also measured. Briefly, the intersection points of the line through the bilateral antegonions on the soft tissue surface were defined, and the length between the soft tissure surface and antegonion was measured as the soft tissue (Fig 1, *D-F*).

All measurements were analyzed by 1 investigator (S.F.) at 3 times, and the same analysis was repeated on another day (with a 2-month interval). and measurement errors were examined. The measurement errors were 0.036 and 0.031 mm for mandibular length and soft tissue thickness, respectively.

Serial undecalcified frozen sections (7 μm thick) were prepared from the frozen mandibular specimens.[22] The sections were fixed with 100% ethanol for 1 minute. The specimens for real-time reverse transcription polymerase chain reaction (RT-PCR) were obtained from the sections by laser capture microdissection using the PALM. MicroBeam system (P.A.L.M. Microlaser Technologies, Bernried, Germany) (Fig 2).[23]

RNA was extracted from the microdissected specimen tissues using the RNeasy Micro Kit (Qiagen, Hilden,

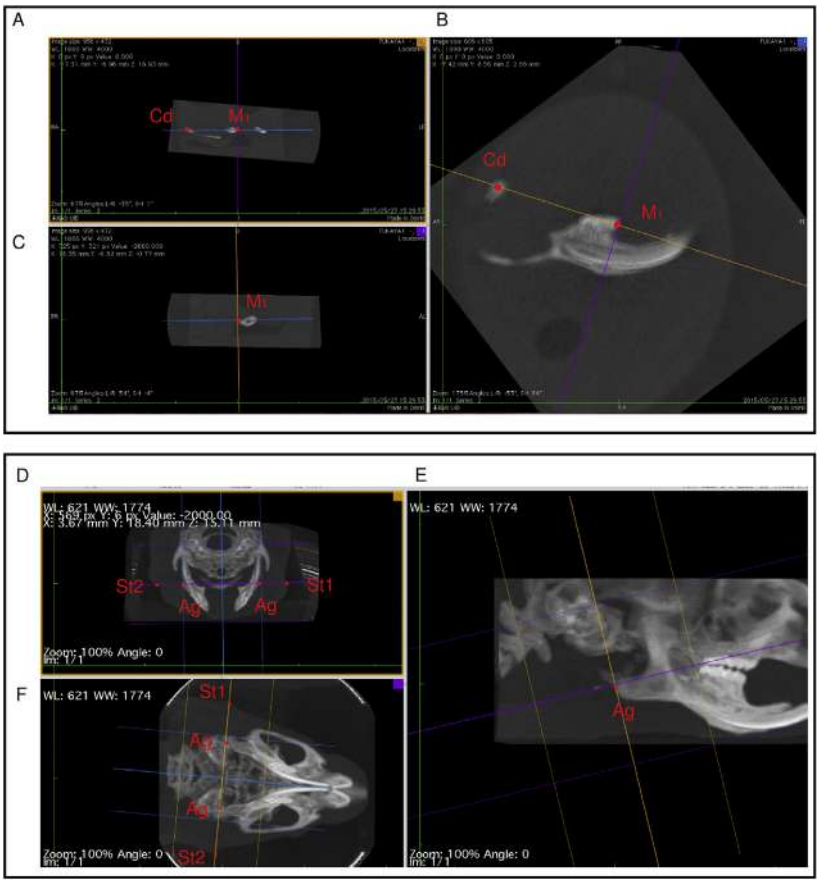

Fig 1. MicroCT analysis: the length from the mandibular first molar to the mandibular condyle was measured. Reference points for the measurement of mandibular length. **A,** Representative images of x-y plane; **B,** y-z plane; and **C,** x-z plane are shown. Measurement of soft tissue thickness at the cheek: microCT analysis of the thickness from the sagittal plane to the soft tissues. **D,** Representative images of y-z plane; **E,** s-y plane; and **F,** x-z plane are shown. Cd, Condylion; M1, first molar; AG, antegonion; St1, St2, surface of the soft tissue.

Germany), according to the manufacturer's instructions. Isolated RNA was reverse transcribed with iScript Reverse transcription supermix (Bio-Rad Laboratories, Hercules, Calif). Real-time RT-PCR analysis was performed with SsoFast EvaGreen Supermix (Bio-Rad Laboratories) using the primers described in the Table. Fold changes of genes of interest with the normalization by glyceraldehyde-3-phosphate dehydrogenase were calculated with ΔΔ cycle threshold method.

IGF-1 levels in the serum at 24 hours after local human IGF-1 injection were measured using human IGF-1 ELISA kits (Enzo; Life Sciences, New York, NY) according to the manufacturer's instructions.

For hematoxylin and eosin histomorphometry, the section was fixed with 4% paraformaldehyde and stained with hematoxylin and eosin dyes. The upper portion of the condylar head was divided into 3 areas: anterior, superior, and posterior. Condylar length, condylar cartilage thickness, and subchondral bone length at each area were measured.

For the bone apposition labeling study, 3 mice in the IGF-1 group were used. Briefly, calcein was injected intraperitoneally in the 10-week-old mice (16 mg/kg of body weight), followed by xylenol orange injection (50 mg/kg of body weight) at age 11 weeks. They were killed a week after the xylenol orange injection, and the mandibular condyles were excised out, and the serial undecalcified frozen sections were prepared. The upper portion of the condylar head was divided into 3 areas: anterior, superior, and posterior. The distance was measured from the calcein (green fluorescence) to the xylenol orange (red fluorescence) in each area.

For the immunohistochemistry, the sections were incubated for 30 minutes in 3% hydrogen peroxide to quench the endogenous peroxidase activity and then blocked with horse serum for 60 minutes at room

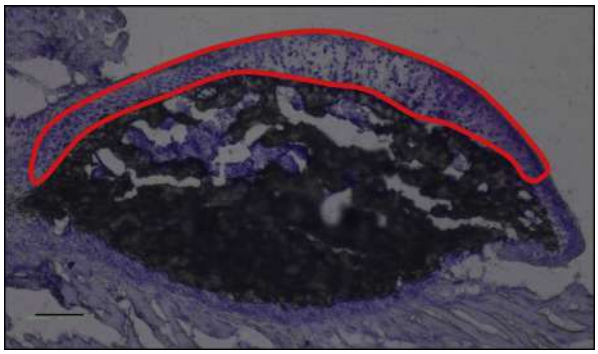

Fig 2. Laser capture microdissection of the condylar cartilage in the frozen section. Whole layers of mandibular condylar cartilage that surrounded the solid line were excised. Bar: 50 μm.

Table. Primer sequences used in this study

Gene	*Accession number*	*Direction*	*Primer sequence (5′ to 3′)*
Col2	NM_031163	Forward	GATGACATTATCTGTGAAG
		Reverse	ATCTCTGATATCTCCAGG
Ihh	NM_010544	Forward	CCTTCATCTTGGTGTAGAGC
		Reverse	GTCCAAAGACAGATGGAATG
Runx2	NM_009820	Forward	GACCTCCAGGAAACCTTTGACAT
		Reverse	GGGCTGGATCTCAAACTCACA
Gapdh	NM_008085	Forward	ACTTTGTCAAGCTCATTTCC
		Reverse	GTGAGGGGAGGAGTCTCAA

temperature. The sections were incubated with the primary antibodies overnight at 4°C, followed by secondary antibodies (ImmPRESS REAGENT Anti-Rabbit Ig; Vector Laboratories, Burlingame, Calif). The primary antibodies were anti Ki67 antibody (1:250 dilution; Santa Cruz Biotechnology, Santa Cruz, Calif), anti Akt-1 antibody (1:200 dilution; Abcam Biochemicals, Cambridge, United Kingdom), and anti type2 collagen (Col2) antibody (1:200 dilution; Rockland Immunochemicals, Gilbertsville, Pa). Immunoreactivity was then visualized using diaminobenzidine and observed with a microscope (BZ-9000; Keyence, Osaka, Japan).

Statistical analysis

All data are given as means and standard deviations. The Student *t* test was used for evaluating statistical significance after testing for normality (version 11.0J; SPSS, Chicago, Ill). $P < 0.05$ was considered statistically significant.

RESULTS

No significant differences in body weight were observed between the IGF-1 and control groups during the experimental period (Fig 3, *A*). There was no significant difference in serum human IGF-1 concentration between the IGF-1 and control groups (Fig 3, *B*).

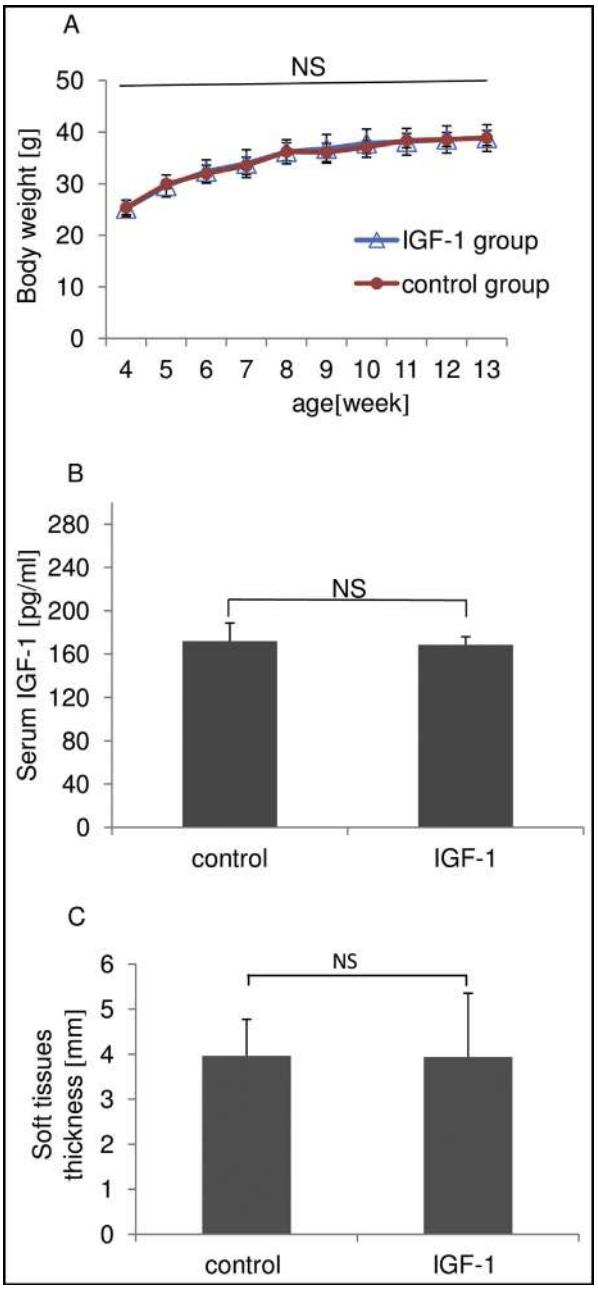

Fig 3. No systemic effect of local unilateral IGF-1 injection was observed: **A,** body weights of the control group (*closed circle*, n = 14) and the IGF-1 group (*open triangle*, n = 35); **B,** serum IGF-1 (*NS*, no significant difference between the groups [control, n = 5; IGF-1, n = 5]); **C,** soft tissue thicknesses at the cheeks are shown (*NS*, no significant difference between the groups [control, n = 8; IGF-1, n = 18]).

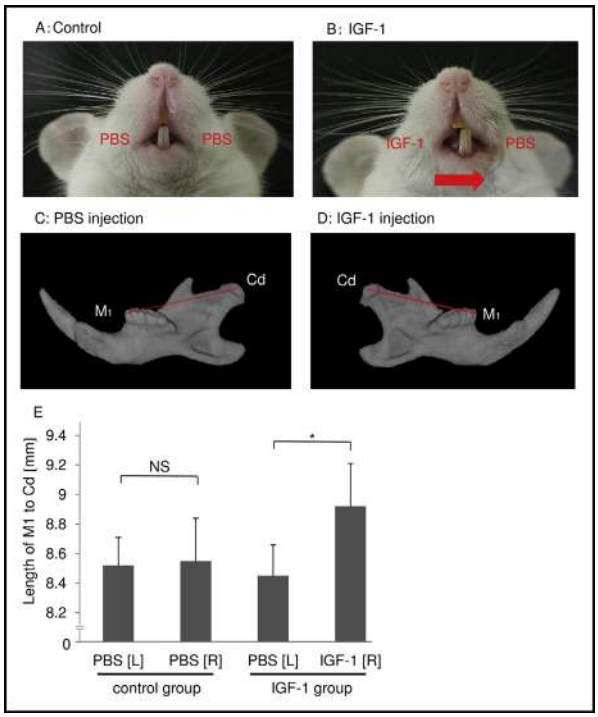

Fig 4. Local unilateral IGF-1 injection induced unilateral growth of the mandible and consequently caused facial asymmetry. Representative facial photographs of **A,** the control group and **B,** the IGF-1 group are shown. Representative microCT images of **C,** the mandible of the PBS injection side and **D,** the IGF-1 injection side are shown. *Cd*, Center of mandibular condyle; *M1*, Mesial contour of the mandibular first molar; **E,** the distance from M1 to Cd is shown. *PBS*, PBS injection side (n = 14); *IGF*, IGF-1 injection side (n = 35); *NS*, no significant difference between the sides. **P* <0.05 between groups.

MicroCT analysis of the soft tissue thicknesses of the cheeks showed no significant differences between the 2 groups (Fig 3, *C*). These results suggest that local unilateral IGF-1 injection into the mandibular condylar cavity had no systemic effects.

Facial symmetry of the mice was assessed at the end of the experiment. Control mice exhibited symmetric mandibular growth with coordination of the upper and lower dental midlines (Fig 4, *A*). On the other hand, in the IGF-1 group, the mandibular midline was deviated to the contralateral side of the IGF-1 injection side (Fig 4, *B*).

These differences suggested that local unilateral IGF-1 injection induces unilateral growth at the mandibular condyle.

In the microCT analysis of mandibular morphology, there were no distinctive differences, such as thickness of the condylar neck and the gonial angle in the lateral view of the microCT images of the mandible between the control (Fig 4, *C*) and the IGF-1 injection sides (Fig 4, *D*) except for condylar morphology. The IGF-1 injection side had a projecting condyle compared with the control side. Then the mandibular length from the mesial contour of the first molar to the condyle was measured. The measurement showed that the mandibular length of the control group was not different between the sides, indicating similar amounts of growth on both sides (Fig 4, *E*). The length of the PBS injection side of the IGF-1 group showed no significant difference compared with that of the control group, signifying no observable effect of the unilateral IGF-1 injection into the contralateral side. Of interest, the mandibular length was significantly longer in the IGF-1 injection side than that in PBS injection side (Fig 4, *E*). These results indicate that local unilateral injection of IGF-1 induced more endochondral growth only in the injected condyle.

The real-time RT-PCR analysis showed that a chondrogenic marker gene, Col2, was distinctively upregulated by local unilateral IGF-1 injection (Fig 5, *A*). In addition, the chondrogenic cytokine, Ihh, was also upregulated (Fig 5, *B*), and the osteogenic marker gene, Runx2, was also upregulated (Fig 5, *C*). These results suggest that local unilateral IGF-1 injection augmented not only chondrogenesis but also osteogenesis.

To further observe the effects of unilateral IGF-1 injection, immunohistochemical staining for IGF-1 signaling molecule, AKT1, proliferation marker, Ki67, and Col2 were performed (Fig 5, *D* and *E*). Many AKT1, Ki67, and Col2 positive cells were identified in the IGF-1 injection side, and a few AKT1, Ki67, and Col2 positive cells were identified even in the cartilage in the PBS injection side. These results suggest that unilateral IGF-1 injection augments IGF-1 signaling, which results in the induction of the increase in chondrogenesis.

To further analyze the induction of endochondral bone formation, histomorphometric analyses were performed using hematoxylin and eosin stained sections (Fig 6). The condylar length was significantly larger in the IGF-1 injection side compared with the PBS injection side (Fig 6, *C*). The condylar cartilage thickness was also significantly greater in the IGF-1 injection side, except for the superior area (Fig 6, *D*). The subchondral bone length was also larger in the IGF-1 injection side (Fig 6, *E*).

Vital labeling by both calcein and xylenol orange showed that the mice had a certain amount of growth at the mandibular condyle between 11 and 12 weeks of age even in the control side (Fig 7, *B*). Compared with the control side, the distance between the labels was increased in the IGF-1 injection side (Fig 7, *C*). Then the mandibular condylar cartilage were divided

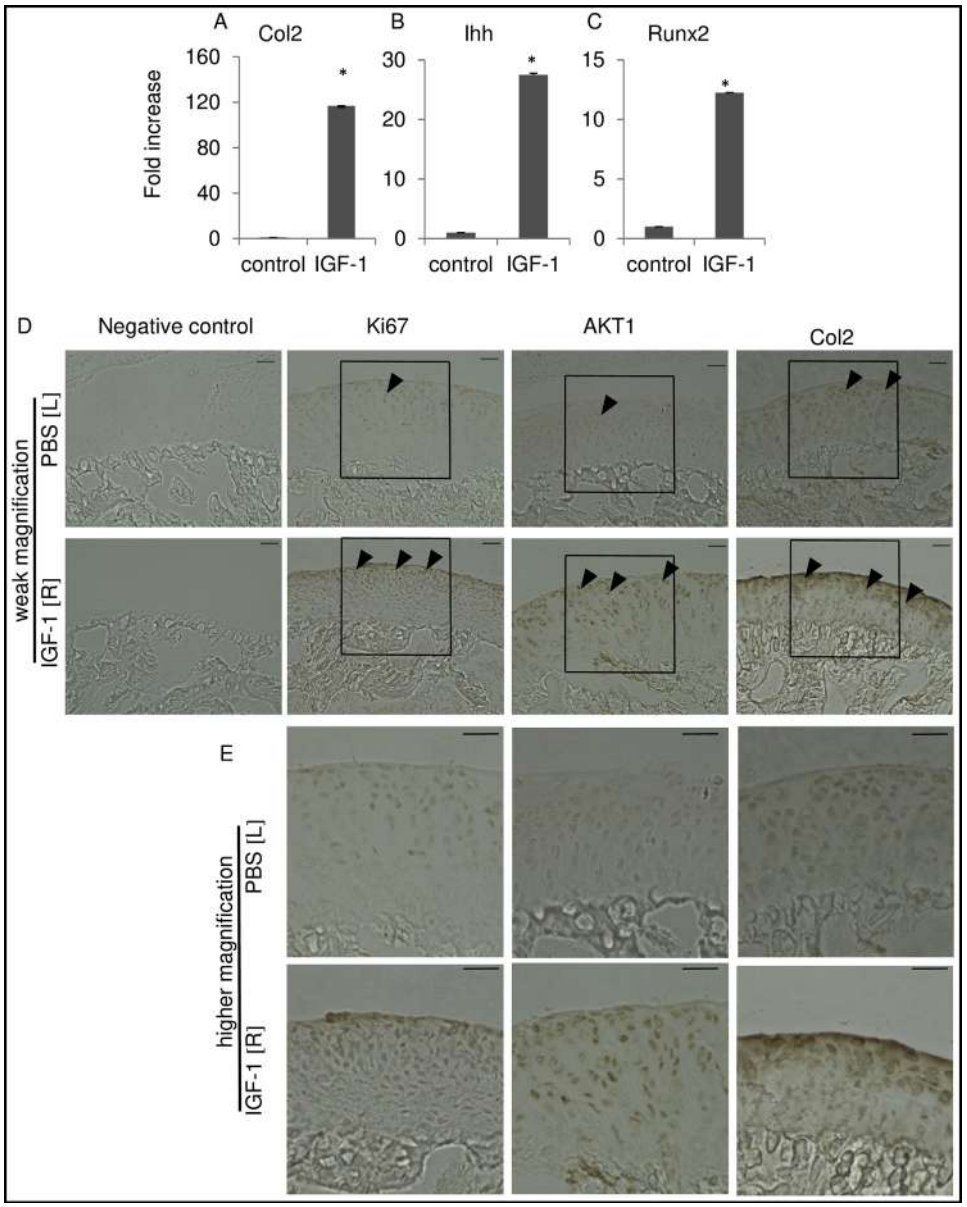

Fig 5. Local unilateral IGF-1 injection induced unilateral endochondral growth of the condyle. The results of real-time RT-PCR analysis of **A,** Col2; **B,** Ihh, and **C,** Runx2 are shown. *$P < 0.05$ (control, n = 4; IGF-1, n = 4). **D** and **E,** Immunohistochemical staining of condylar cartilage (control, n = 3; IGF-1, n = 3). Representative images of Ki67 (second from left), AKT1 (third from left), and Col2 (right side) are shown in the weak (**D**) and higher (**E**) magnifications. In each staining, the upper panels are photographs of the PBS injection side, and the lower panels are the IGF-1 injection side. The left-side photographs indicate negative control, and the other photographs indicate immunoreactivity visualized with diaminobenzidine. Bar: 30 μm.

into 3 areas—anterior, superior, and posterior (Fig 7, *A*)—and measured at each area. Increases in endochondral bone formation by local unilateral IGF-1 injection were observed at all 3 areas (Fig 7, *D*). The anterior area exhibited remarkable induction.

DISCUSSION

In this study, local unilateral IGF-1 injection into the mandibular condylar cavity successfully induced acceleration of unilateral endochondral mandibular growth on the injection side with no systemic adverse effects

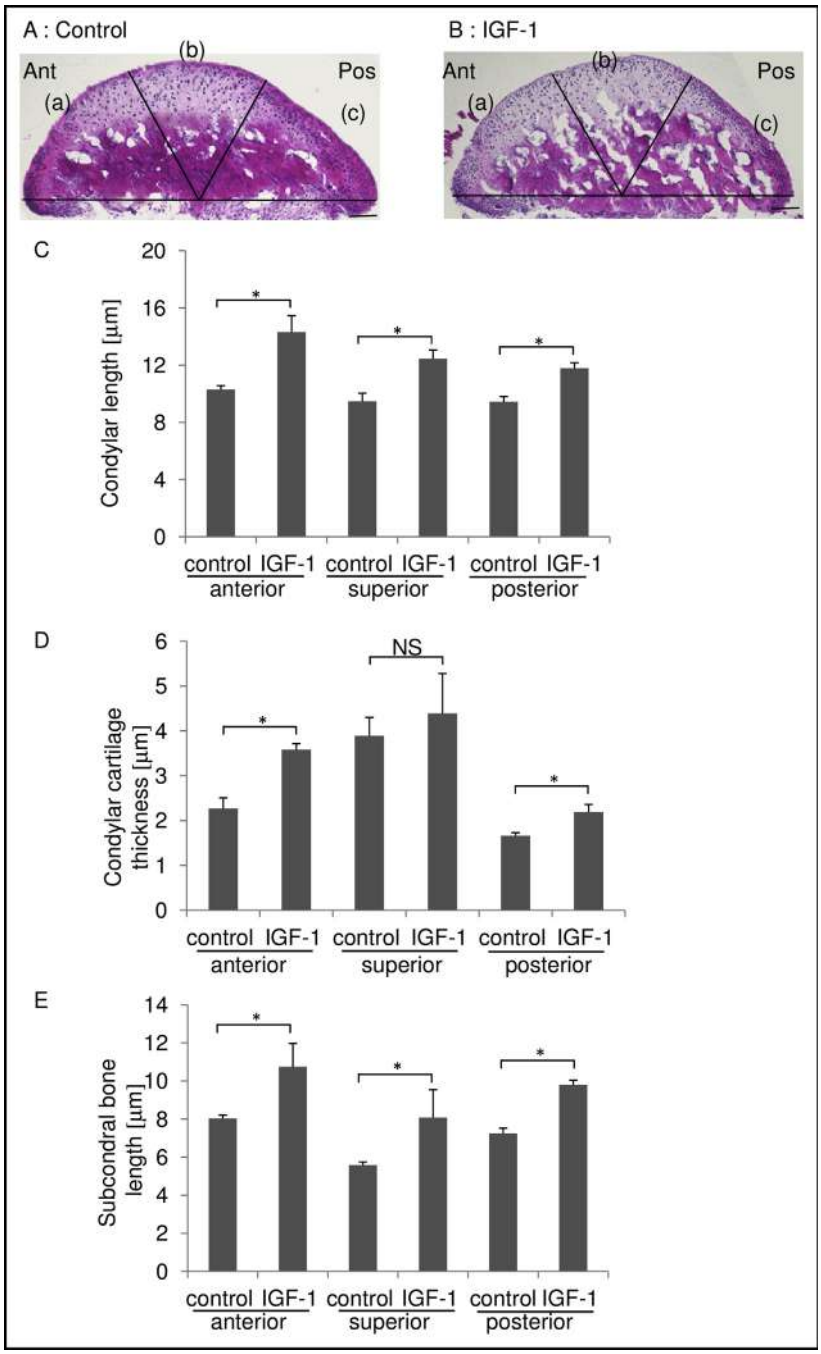

Fig 6. Histomorphometric analysis of hematoxylin and eosin stained sections. Photographs of stained mandibular condylar heads in **A,** control group (n = 3) and **B,** IGF-1 group (n = 3) are shown. Bar = 200 μm. *Ant*, anterior; *Pos*, posterior. The condylar head was divided into 3 areas: anterior, superior, and posterior. The convergent point of the borders to each area was used as the reference point for measuring the condylar length and the subchondral bone length. **C,** Condylar length; **D,** condylar cartilage thickness; and **E,** subchondral bone length are shown. *$P < 0.05$; *NS*, no significant difference between the groups.

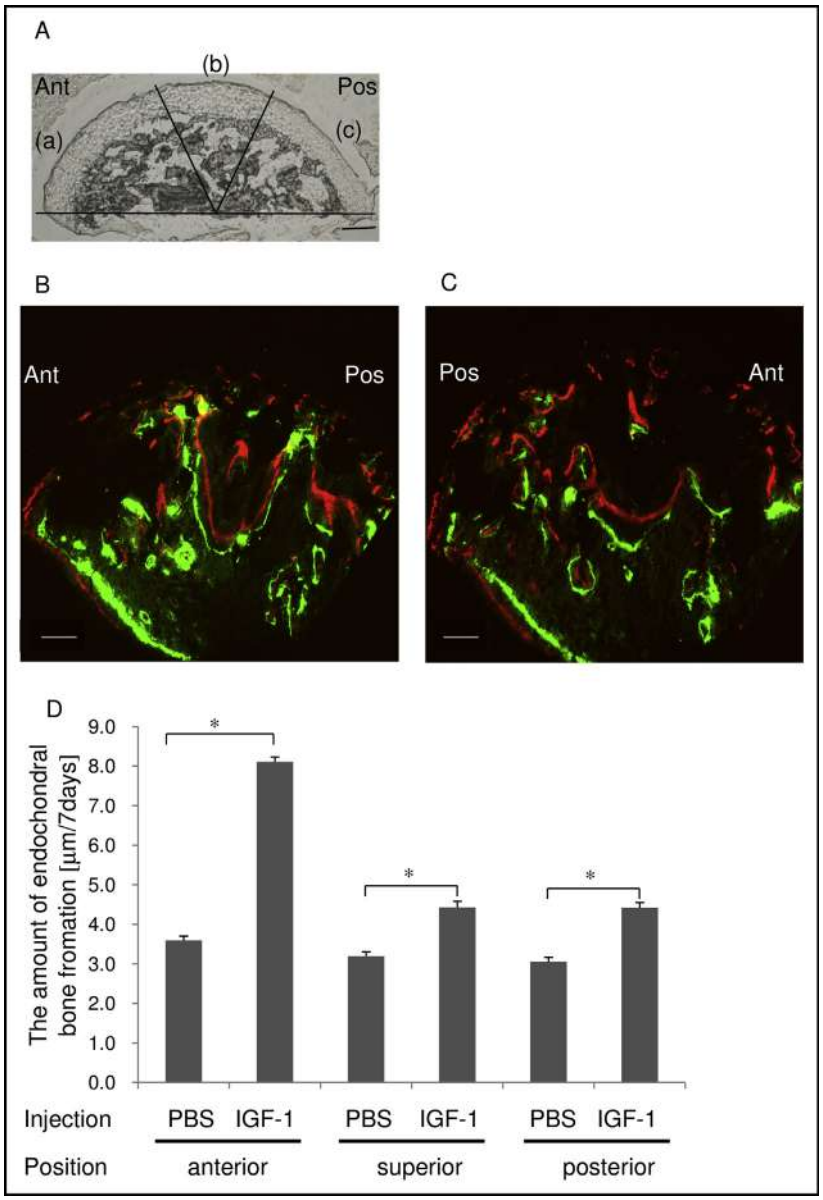

Fig 7. Local unilateral IGF-1 injection induced the distance between vital staining of calcein (*green*) and xylenol orange (*red*). **A,** Bright-field photograph of mandibular condylar head. Bar = 200 μm. *Ant*, anterior; *Pos*, posterior. The condylar head was divided into 3 areas: anterior, superior, and posterior. Representative fluorescent microscopic images of the condyle of **B,** the PBS injection side (n = 3) and **C,** the IGF-1 injection side (n = 3) are shown. Bar: 100 μm. **D,** The amount of endochondral bone formation during 7 days. Comparisons of IGF-1 injection side and PBS injection side in each area are shown. **P* <0.05.

in the mice. In previous reports, systemic administration of exogenous IGF-1 or local injection into the bilateral mandibular condylar cavities accelerated growth of the condylar cartilage.[16,17] However, there was no report about the effect of local unilateral IGF-1 injection into the mandibular condylar cavity. To develop a therapeutic approach for mandibular asymmetry in the future, we chose local unilateral IGF-1 injection into the mandibular condylar cavity. Conventional unilateral growth stimulation using appliances or other methods are common.[8-10,24] Further validation in larger experimental animals is necessary to compare the effect of the local unilateral IGF-1 injection into the mandibular condylar cavity with that of a functional appliance.

It is well known that symmetry of maxillofacial bone is essential for an ideal occlusion.[1,2] Clinically, functional appliances would be applied in patients with mandibular asymmetry.[8-10,24] However, the effects of functional appliances depend on the patients' compliance; therefore, it is difficult to predict the results.[25] Local unilateral IGF-1 injection obtained the desirable effect without compliance and systemic adverse effects. The mandibular condylar cavity is surrounded by articular capsules, which make it possible to maintain higher local concentrations of injected IGF-1 in the mandibular condylar cavity.[6] There were no differences in serum IGF-1 levels or body weights between the control group and the IGF-1 injection group. Furthermore, there was no effect on the soft tissue thickness of the cheeks. Consistent with previous reports, local IGF-1 injection into the mandibular condylar cavity had no systemic adverse effects.[19,20] Therefore, local unilateral injection of cytokine into the mandibular condylar cavity is beneficial for the correction of mandibular asymmetry during the growth period.

MicroCT analysis was performed under unblinded conditions, and there still remained a possibility of cognitive bias in the microCT analysis. However, the differences in the increases in the endochondral growth and ossification were quite evident between the IGF-1 injection and the PBS injection sides in the IGF-1 group; this suggests that there is little influence of cognitive bias on the results in the analysis.

Itoh et al[19] showed the augmented endochondral mandibular growth by local bilateral injection of IGF-1 into the mandibular condylar cavity. It is known that Akt1 is 1 intracellular signaling molecule after IGF-1.[26] Immunostaining clearly indicated that local unilateral IGF-1 injection induced IGF-1 signaling via Akt1. Furthermore, local injection of IGF-1 into the mandibular condylar cavity accelerated the growth of the chondrocytes in mandibular condylar cartilage, which led to an increased cartilaginous matrix. Chondrocyte proliferation was induced by IGF-1 injection judged by immunostaining for Ki67, which was used as a marker in cell proliferation and cell cycle studies.[27]

In our study, the induction of endochondral bone formation was remarkably large at the anterior area compared with the other 2 areas of the condyle. However, in previous research, it was reported that the effect of extrinsic force on mandibular condylar growth in young rats showed a tendency to increase significantly in the posterior area.[28] Regarding the expression of IGFR, it was reported that the posterior area exhibited few IGFR expressions in rats.[29] Taken together, we presumed that the stronger effect of unilateral IGF-1 injection on endochondral growth induction at the anterior area would be due to the abundance of IGFR at the anterior area.

It is known that the main extracellular matrix component in the mandibular condylar cartilage is collagen.[27,30] In the previous report, upregulation of Col2 was observed by local injection of IGF-1 and TGF-β into bilateral mandibular condyles in rabbits.[31] Our results also showed that increased Col2 expression was induced in the IGF-1 injection side, specifically indicating the augmentation of cartilage formation by the local injection of IGF-1.

Not only chondrogenic genes but also the osteogenic gene, Runx2, was upregulated by IGF-1 injection.[32] As Tang and Rabie[33] reported, Runx2 regulates endochondral ossification in the condyles; our results are consistent with their report.

Although the local unilateral IGF-1 injection into the mandibular condylar cavity successfully induced unilateral endochondral growth of mandible in mice, further large animal testing and clinical research involving dosing tests, application intervals, and a number of other variables should be performed before progress to clinical applications.

CONCLUSIONS

These results verified that local unilateral IGF-1 injection into the mandibular condylar cavity successfully induced unilateral endochondral mandibular growth in mice without systemic adverse effects. Thus, local unilateral IGF-1 injection into the mandibular condylar cavity could be a useful alternative for mandibular asymmetry therapy during the growth period. However, additional experimental and clinical studies are necessary to prove the real effect of this new therapy.

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