bFGF の皮下注射は授乳期における新生児マウスの下顎頭の発育を促進する

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Subcutaneous Basic FGF-injection Accelerates the Development of Mandibular Condyle of Newborn Mice during Lactation Period

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【目的】

　線維芽細胞増殖因子(FGF)は，ウシ下垂体から精製された成長因子であり，種々の細胞の増殖や分化に関与する．bFGF(FGF-2)は軟骨組織の恒常性維持や成長発育に重要なであり，下顎頭でも正常組織だけでなく腫瘍細胞の増殖促進に関与することが知られている．ラット膝関節へのbFGF局所投与実験はあるが，下顎頭におけるbFGFの局所作用に関する報告は知られていない．そこで，本研究では新生児マウスの下顎頭におけるbFGFの局所投与効果を検討した．

【方法】

　実験動物は，生後 0 日齢の ddY 系マウス 40 匹を用いた．実験群は，1μg/μl の rhbFGF を生後 1 日目，2 日目，3 日目の 3 回(0.1μl/回)に分けて左側下顎頭に総量 0.3μg 投与した．対照群は，右側下顎頭に同量の生理食塩水を局所投与した．投与 1 日後，4 日後，7 日後および 14 日後に各群 10 匹を屠殺し，4%パラホルムアルデヒドに浸漬固定した．通法に従って10%EDTA で脱灰後，パラフィン包埋し，3μm の前頭断連続切片(約 800 枚／ブロック)を作製し，20 枚毎に HE 染色を行った．下顎頭が最大幅径となる標本を含む20枚の連続切片を用いてPCNA免疫染色を行った．HE 標本では下顎頭の最大幅径，増殖層の厚さおよび下顎頭の長さに対する増殖層の長さの比率を求めた．PCNA 免疫染色標本では，増殖層の細胞数に対する PCNA 陽性細胞の割合を求めて増殖率(PI)とした．形態計測と陽性細胞数の抽出には Motic® Images Plus 2.2 ver.2.1.2 を用いた．これらの結果は，Wilcoxon signed-rank test で対照群と実験群を比較した．群間の多重比較は Kruskal-Wallis test を用い，Steel-Dwass test で事後検定した．全ての統計解析は R で行い $p<0.05$ を有意差ありとした．

【結果】

　1. 組織学的所見：対照群と実験群の下顎頭に明らかな構造異常はみられなかった．投与 1 日後では，実験群で増殖層の著しい肥厚が確認された．投与後 4 日でも同様に増殖層の肥厚を認め，さらに軟骨細胞と肥大軟骨細胞の数は増加し，最大幅径と垂直方向の増加があった．投与 7 日後の対照群では頭頂から骨化層までの垂直距離が短縮し、骨化層先端部は下顎頭方向へ移動していた．しかし，実験群では対照群に比較して軟骨組織の全体的な厚さは依然として厚かった．投与後 14 日では，実験群でも対照群同様に最大幅径の増加と骨化の亢進があり，骨化層の前線は頭頂方向へ移動していた．

　2. 免疫染色所見：PCNA 陽性細胞は主に増殖層に分布し，対照群に比べ実験群で陽性率が高い傾向を示した．投与後 7 日までは陽性細胞は増加したが，実験 14 日目で陽性細胞は減少していた．

　3. 形態計測解析：対照群の最大幅径は 408.0±128.3μm(1 日後)，512.8±25.5μm(4 日後），629.4±36.8μm（7 日後），743.3±78.7μm（14 日後）であり，実験群は，533.4±86.1μm（1 日後），512.8±25.5μm（4 日後），722.6±56.7μm（7 日後），810.8±90.8μm（14 日後)だった．最大幅径は対照群と実験群ともに徐々に増加する傾

向を示し，統計学的に有意に実験群の最大幅径が大きかった（$p<0.01$）．増殖層の厚み
は，対照群で 117.1±4.4μm（1 日後），124.5±6.0μm（4 日後），136.5±3.4μm（7 日後），
129.9±2.4μm（14日後）であり，実験群は155.0±6.7μm（1 日後），195.2±8.2μm（4 日後），
175.8±10.3μm（7 日後），142.1±8.0μm（14 日後）だった．対照群は7日目まで徐々に増
加した後に減少傾向を示し，実験群は4日目に最大値を示し徐々に減少する傾向を示
した．実験群の4日目が有意に他群に比べ厚かった．増殖層の厚さの比率は，対照群
で 50.5±2.0%（1 日後），49.8±3.0%（4 日後），49.4±1.0%（7 日後），36.2±1.0%（14 日後）
であり，実験群で 60.4±1.0%（1 日後），69.7±3.0%（4 日後），58.6±4.0%（7 日後），
37.8±2.0%（14日後）だった．対照群は，7日目まで変化は無く14日目に値が小さくなっ
た．実験群は，増殖層の厚さと同様に4日目に最大値を示し，その後減少した．実験群
の4日目の値は他群に比べ有意（$p<0.001$）に高値であった．

　4．増殖率解析：PIは，対照群で 39.7±3.0%（1 日後），43.2±1.0%（4 日後），
47.1±3.0%（7 日後），43.0±3.0%（14 日後）で，実験群では 50.2±3.0%（1 日後），
68.9±4.0%（4 日後），50.5±3.0%（7 日後），50.4±2.0%（14 日後）だった．対照群の増殖
率は1日目から徐々に増加傾向を示し7日目に最大となった．実験群は，4日目に最大
値を示し統計学的にも有意（$p<0.001$）に高値だった．

【考察】

　増殖層の厚さと増殖率は，対照群が7日目，実験群では4日目に最大となった．これ
らはrhbFGFが間葉系細胞の増殖を促進した結果と考えられた．しかし，rhbFGFは生体
内で24時間以内に消失するので，実験群が4日目で効果が最大となる結果と矛盾する．
生体内では bFGF はbFGF と TGF-β1 の発現を増強する。さらに TGF-β1 もbFGF の発
現を増加することから，成長因子の相乗作用により効果時間が延長し，投与後4日目に
最大の効果が現れたものと考えられた．また，実験群の軟骨細胞の層が厚く，線維層か
ら骨化層先端までの距離が長かったことから，rhbFGF は軟骨細胞や肥大軟骨細胞へ
の分化を促進するが，軟骨細胞の最終分化を抑制している可能性が示唆された．本研
究は，rhbFGF の局所投与が授乳期マウスの下顎頭間葉系細胞の増殖を促進し得るこ
とを示した．

Original

Subcutaneous Basic FGF-Injection Accelerates the Development of Mandibular Condyle of Newborn Mice during Lactation Period

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Abstract: To elucidate the effect of local bFGF administration, we administered a total of 0.3μg rhbFGF was to the left mandibular condyle by three injections of 0.1μl bFGF solution for 3 days after birth. The contralateral condyles with three injections of 0.1μl physiologic saline served as controls. Serial sections including the widest condyle were evaluated with H-E staining and immunostaining for PCNA. The maximum width of condyle, proliferative zone thickness, proliferative zone thickness ratio and proliferating index were analyzed using image analysis software after digital image capture of H-E stained sections. All condyles were composed of fibrous, proliferative, maturation and hypertrophic cell zones without significant abnormal findings. Experimental condyles demonstrated a markedly thickened proliferative zone compared with that of the controls at 1 day after the injection. At 4 and 7 days, chondrocytes and hypertrophic chondrocytes of the experiments increased in thickness. However, at 14 days after the injection, both the controls and experiments showed similar features with an upward shift of ossification front and active formation of bone trabeculae. Morphometrically, all values of experiments were significantly higher than those of the controls. Especially at 4 days after the final injection, proliferative zone thickness and proliferative index values of the experiment reached a peak and were nearly 1.6-fold higher than those of the control, which were statistically significant compared to other experimental groups. Despite the short half-life of rhbFGF, its effect seems to be prolonged by synergic actions of growth factors such as bFGF or TGF beta1. In conclusion, we showed that local administration of bFGF was feasible for accelerating mesenchymal cell proliferation of mandibular condyles of newborn mice in the lactation period after birth.

Key words: Basic FGF, Condyle development, Local administration, Newborn mice

Introduction

Fibroblast growth factor (FGF) is a growth factor that was first purified as a polypeptide of 13,300 molecular weight from bovine pituitary gland in 1970s[1]. FGF isolated under non-acidic conditions was referred to as basic pituitary FGF possessing mitogenic activity[2]. However, FGFs are now recognized as polypeptide growth factors of 150 to 250 amino acid residues with myriad biological activities and expression profiles in variable kinds of cells or tissues. These are encoded by 22 kinds of *Fgf* genes (*Fgf*1 to *Fgf*23), while FGF receptors are translated from four members of *Fgfr* gene domain. Most FGFs are secretion proteins that bind to FGFRs and induce the phosphorylation[3, 4]. This family, binding heparin and heparan sulfate, regulates articular cartilage homeostasis and controls the growth,

differentiation, migration and survival of a wide variety of cell types. In this family, basic fibroblast growth factor (bFGF) also known as FGF-2 regulates mesenchymal cell proliferation but not epithelial cell[5]. This regulator plays an important role in cartilage matrix homeostasis[6] and bone growth and development[7]. In mandibular condyle, bFGF modulates cartilage proliferation not only in normal condylar cartilage cells[8] but also in neoplastic cartilage cells such as in synovial chondromatosis[9]. In addition, this factor seems to be physiologically involved in the alteration of the mandibular condylar cartilage due to lateral movement of the mandible[10].

Clinically, bFGF has been applied to various diseases. For example, it is effective in wound healing and has been applied in cases of plastic surgery[11]. There has been considerable research on the treatment of articular disorders. Although the trial of bFGF injection into rat knee joints had been reported by Shida and his coworkers[12], there is no *in vivo* investigation of the effect on local

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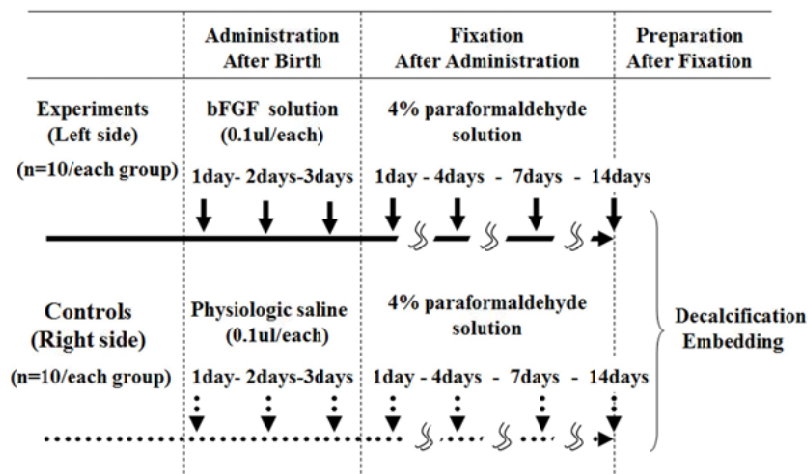
Figure 1. Experimental design of rhbFGF injection into the mandibular condyle of newborn mice

bFGF injection into mandibular condyles. Our goal of current study is to elucidate the effect of local bFGF administration on newborn mouse condyles.

Materials and Methods

Animal and experimental preparations

Forty newborn ddY mice (Japan SLC Inc. Hamamatsu, Japan) were divided into four groups (10 /group). Mice weighed approximately 1 g at birth and 6 g or more at 2-weeks of age. Prior to experiments, we prepared handmade micro-syringes that enabled us to eject 0.1 μl solution per one drop using a NanoPass® tapered 33 gauge needle (Terumo, Tokyo, Japan). To confirm the position of the needle tips and distribution of injected solution, 3D micro-CT (R mCT, Rigak, Tokyo, Japan) images were taken before and after subcutaneous injection of contrast medium (Iopamiron® Inj., Bayer Yakuhin, Ltd., Tokyo, Japan).

Experimental design

Ten μg of recombinant human basic fibroblast growth factor (rhbFGF, PROGEN Biotechnik GmbH, Heidelberg, Germany) was reconstituted in 10 μl distilled water. Final concentration of bFGF solution was 1 μg/μl. As shown in Fig. 1, total 0.3 μg of rhbFGF was administered to the left mandibular condyle by three injections of 0.1 μl bFGF solution for 3 days after birth. The contralateral, right condyle received three injections of 0.1 μl physiologic saline. The left and right mandibular condyles served as experiments and controls, respectively. The new bone mice were kept in an air-conditioned room with controlled temperature at 24±1ºC and fed with their mother, freely taking solid feed (Japan SLC Inc. Hamamatsu, Japan) and water. The ethics committee on laboratory animals of Matsumoto Dental University approved this study.

Preparation and selection of histological sections

Ten mice of each control and experimental group were euthanized at 1, 4, 7 and 14 days after the final injection. Resected specimens were routinely treated after fixation in 4% paraformaldehyde in 0.05 M phosphate buffer for 24 hours and decalcification in 10% EDTA for 3 weeks. Three micron-thick, serial frontal sections through both condyles were made from paraffin blocks. About 800 sections including both condyles were obtained from each sample.

In order to select the section that contained the widest frontal section of the condyle, one out of every 20 sections was stained with hematoxylin and eosin (H-E) and the condyle widths of both sides were measured using a microscope system (Olympus BX61 with digital camera DP71, Olympus Corporation, Tokyo, Japan) and Motic Image Plus 2.1 version 2.1.2 (Shimazu, Tokyo, Japan). Finally, we used a set of 20 serial sections including the widest section for immunohistochemical staining.

Immunohistochemistry

After high-temperature unmasking technique was performed by an autoclave at 121°C for 15 minutes in 0.01 M sodium citrate buffer solution (pH6.0), three micron-thick paraffin sections were routinely treated. Anti-proliferation cell nuclear antigen (PCNA) antibody (Dako, Glostrup, Denmark) as primary antibodies and Nichirei MAX-PO Multi (Nichirei, Tokyo, Japan) as a secondary antibody were incubated 4℃ overnight and at room temperature 30 minutes, respectively. After visualization with 3-3'-diaminobenzidine tetrahydrochloride (Dako, Glostrup, Denmark), sections were counterstained with hematoxylin. Negative control slides were processed without the primary antibodies.

Image analyses

All digital images (JPEG, 1600×1200 pixels) of H-E staining sections were captured with a 10× objective lens using a microscope system (Olympus BX61 with digital camera DP71, Olympus Corporation, Tokyo, Japan). As is shown in Fig. 2, values of maximum width, proliferative zone thickness and condylar
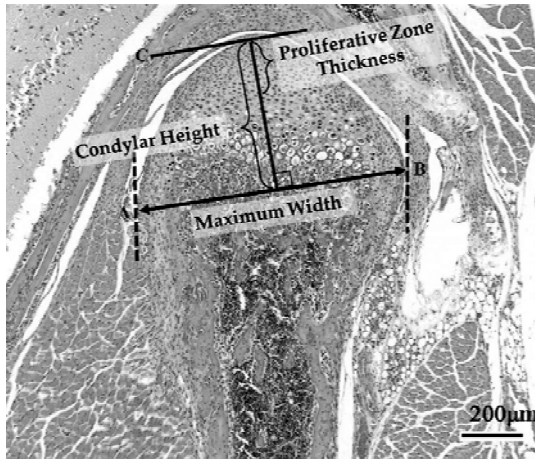
Figure 2. Schematic illustration of morphometric analysis
Line A and B are tangents parallel to the longitudinal axis of the condyle. Line C is the tangent to the top of condyle which is parallel to the line AB. To measure proliferative zone thickness and condylar height, a perpendicular line was drown from the contact point of line C.

height were histomorphometrically analyzed using Motic Image Plus 2.1 version 2.1.2 (Shimazu, Tokyo, Japan). Briefly, maximum width was defined as the distance between parallel tangents to the axis of condylar head. Proliferative zone thickness, defined as the distance from the top of the condyle to the end of proliferative zone including fibrous and proliferative zones, was measured along the perpendicular line to the measurement line of maximum width. Condylar height was measured as the distance from the top of condyle to the cross point between the perpendicular line and maximum width measurement line. Proliferative zone thickness ratio (%) was calculated as proliferative zone thickness divided by condylar height.

In order to analyze proliferating index, captured JPEG files (1600×1200 pixels) were processed with Motic Image Plus 2.1 version 2.1.2 (Shimazu, Tokyo, Japan). Briefly, we selected arbitrary areas in proliferative and differentiated zones including more than 800 and 1,000 cells, in controls and experiments, respectively. PCNA-positive and negative nuclei, namely brown- and blue-stained nuclei, were extracted spectrally and automatically counted. The percentage of positive cells divided by the sum of positive and negative cells was calculated as proliferating index (PI).

Statistical analyses

After calculating the Shapiro-Wilk-test for normality checking, the comparison of histomorphometric analysis data between controls and experiments was analyzed with Wilcoxon signed-rank test. Multiple comparisons of values among four groups of controls and experiments were analyzed using the Kruskal-Wallis test followed by Steel-Dwass test. All statistical analyses were performed with R (The R Foundation for Statistical Computing,

version 2.13.0). Simultaneously, *p* values less than 0.05 were considered statistically significant.

Results

Macroscopic findings

All animals of both controls and experiments showed neither mandibular malposition nor craniofacial malformations. The skin of the treated area demonstrated no dermal lesions such as dermatitis or ulcerative change.

Histological findings

One day after the final bFGF injection, control condyles were covered by fibrous tissue namely fibrous or articular zone composed of some collagen fibers and fibroblasts. Beneath the fibrous zone, a thin highly cellular area or proliferative zone of mesenchymal spindle cells was noted, followed by a maturation zone comprising flatten or oval chondrocytes embedded in hyaline matrix. Finally, large chondrocytes were distributed in the hypertrophic cell zone in the deepest area (Fig. 3a). Experimental condyles showed neither traumatic changes nor morphological abnormalities in each layer. Fibrous to hypertrophic cell zones were clearly recognized without frank hyperplasia. However, fibrous to proliferative zones were markedly thicker than those of the control (Fig. 3b).

Four days after the final injection, features of controls showed similar to that of the control at 1 day after the injection. The number of mesenchymal cells of proliferative zone and the thickness of the fibrous zone to hypertrophic cell zone somewhat decreased (Fig. 3c). In experiments without any abnormal formations, the proliferative zone somewhat reduced its thickness comparing to that of the 1-day-experimental sample, while, chondrocytes and hypertrophic chondrocytes increased in number. Simultaneously, the distance of the condylar top to erosive zone and the maximum width of the experiment were greater than those of the control condyle (Fig. 3d).

Seven days after the injection, controls showed that the distance from the fibrous zone to the front of endochondral ossification markedly decreased compared with that of the 4-day group (Fig. 3e). Seven-day experiments showed that proliferative, maturation and hypertrophic cell zones were somewhat thicker than those of controls without any abnormal morphology. The front of ossification was moved toward the condylar top in contrast to the 4-day experiments, but this boundary was away from the top of the condyle compared with the controls (Fig. 3f).

Fourteen days after the injection, both the controls and experiments showed similar morphological features, and significantly increased in width compared with the 7-day group. The thickness of fibrous to hypertrophic cell layers became somewhat thin, accompanied by the upward shift of ossification front and active formation of bone trabeculae (Fig. 3g, 3h). This
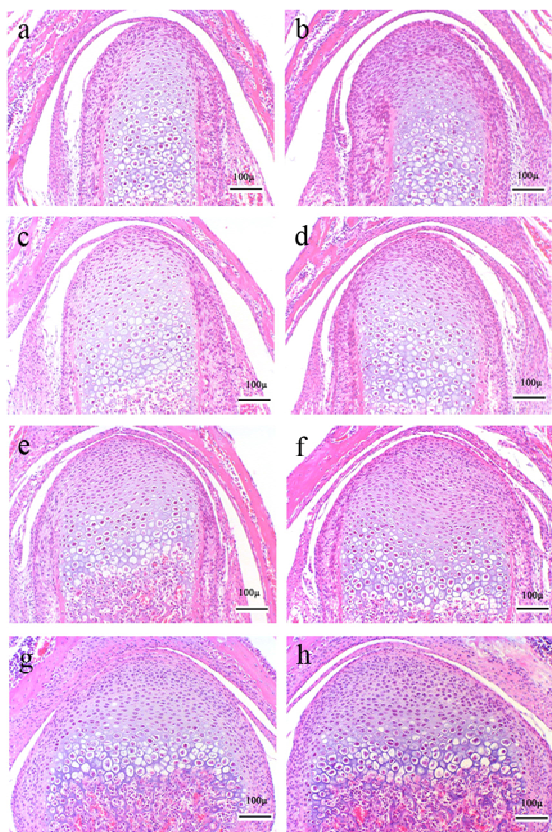
Figure 3. Histological features of controls at 1, 4, 7 and 14 days after the injection (a, c, e, g) and experiments at 1, 4, 7 and 14 days after the injection (b, d, f, h): Condyles of both controls and experiments gradually increase in width with the time course. Note the difference between thickness of mesenchymal and cartilaginous cells of the control and experiment especially at 7 days after the injection (e and F).
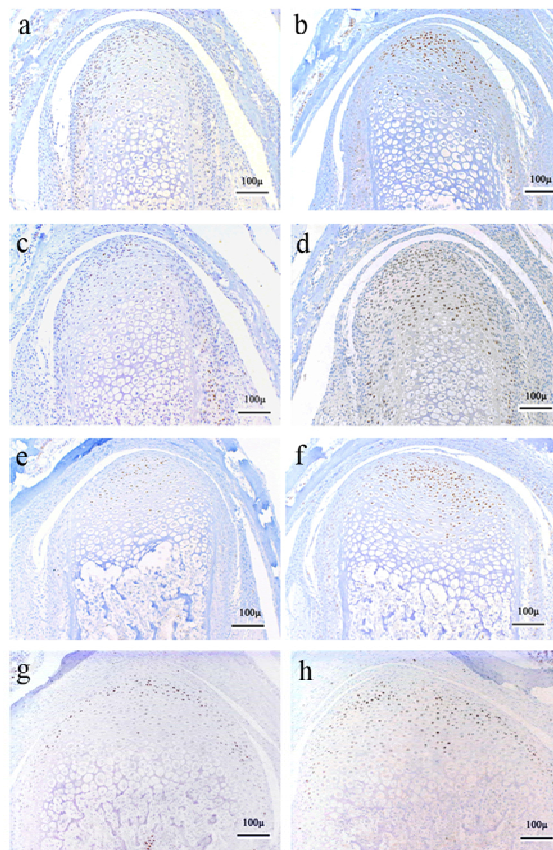

Figure 4. PCNA-immunostaining features of controls at 1, 4, 7 and 14 days after the injection (a, c, e, g) and experiments at 1, 4, 7 and 14 (b, d, f, h). Positive cells of experiments are more than those of experiments.

change was remarkable in the experiments (Fig. 3 3h).

Immunohistochemical findings

PCNA-positive cells were mostly distributed in the proliferative zone but a few positive cells were also observed in the maturation zone in all groups. Positive cells of experimental groups were more than those of controls. Experimental groups tended to gradually increase in number from the 1-day group to the 7-day group. However, positive cells of the 14-day group seemed to decrease in number in comparison with the 7-day group (Fig. 4).

Histomorphometric analysis

Morphometric data are summarized in Table 1. Maximum width values of both controls and experiments gradually increased with the time course (408.0 ± 16.5 μm and 533.4 ± 86.1 μm at 1 day, 512.8 ± 25.5 μm and 674.2 ± 128.3 μm at 4 days, 629.4 ± 36.8 μm and 722.6 ± 56.7 μm at 7 days, 743.3 ± 78.7 μm and 810.8 ± 90.8 μm at 14 days, in controls and experiments, respectively). Maximum width values of experiments were significantly higher ($p<0.01$) than those of controls. Experimental values were 1.31,

1.31, 1.15 and 1.09 fold larger than those of controls at 1 day, 4 days, 7days and 14 days after the injection, respectively (Table 1). Among experimental values, there were statistical differences except for the 4-day and 7-day groups, the 4-day and the 14-day groups and the7-day and 14-day groups (Fig. 5a).

Proliferative zone thickness, namely the thickness of fibrous and proliferative zones, showed a significant difference between controls and experiments: 117.1 ± 4.4 μm and 155.0 ± 6.7 μm at 1day, 124.5 ± 6.0 μm and 195.2 ± 8.2 m at 4 days, 136.5 ± 3.4 μm and 175.8 ± 10.3 μm at 7 days, 129.9 ± 2.4 μm and 142.1 ± 8.0 μm at 14 days, in controls and experiments, respectively. In controls, proliferative zone thickness gradually increased and reached to a peak at 7 days after the injection and turned to decline, while experiments exhibited the maximum value at only 4 days after the injection. Experimental values were 1.32, 1.57, 1.29 and 1.09 fold larger than those of controls at 1 day, 4 days, 7days and 14 days after the injection, respectively (Table 1). Multiple comparison tests revealed statistically significant differences among all experimental and control groups except for between the 4-day and 14-day controls (Fig. 5b).

The trend of proliferative zone thickness ratio was similar to that of proliferative zone thickness. All experimental ratios were
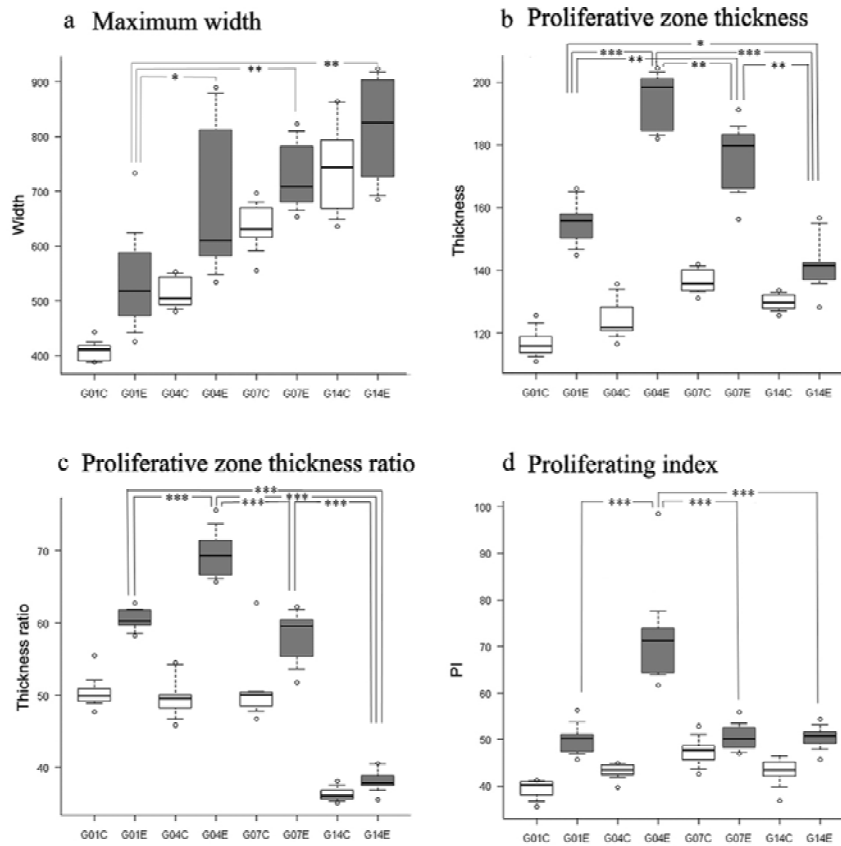
Figure 5. Box plots of maximum width (a), proliferative zone thickness (b), proliferative zone thickness ratio (c) and proliferative index (e). White and gray columns show controls and experiments, respectively. Width: maximum width of condyle, Thickness: proliferative zone thickness, Thickness ratio: proliferative zone thickness ratio, PI: proliferating index, G01C/E, G04C/E G07C/E and G14C/E: 1-day, 4-day, 7-day and 14-day groups of the controls and experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$, Kruskal-Wallis test with Steel-Dwass test

Table 1 Histomorphometrically analyzed data of controls and experiments

		1 day	4 days	7 days	14 days
Cont vs. Exp	MW, μm	408.0 vs. 533.4**	512.8 vs. 674.2**	629.4 vs. 722.6**	743.3 vs. 810.8**
	PT, μm	117.1 vs. 155.0**	124.5 vs. 195.2**	136.5 vs. 175.8**	129.9 vs. 142.1**
	Tratio	50.5 vs. 60.4**	49.8 vs. 69.7**	49.4 vs. 58.6**	36.2 vs. 37.8**
	PI	39.7 vs. 50.2**	43.2 vs. 68.9**	47.1 vs. 50.5*	43.0 vs. 50.4**

MW: maximum width of condyle, PT: thickness of fibrous and proliferative zones, T ratio: PT divided by condylar height, PI: proliferating index, *$p<0.05$, **$p<0.01$, (Wilcoxon signed-rank test)

significantly higher ($p<0.01$) than those of controls: 50.5±2% versus 60.4±1% at 1day, 49.8±3% versus 69.7±3% at 4 days, 49.4±1% versus 58.6±4% at 7 days, 36.2±1% versus 37.8±2% in controls and experiments, respectively. Experimental values were 1.20, 1.40, 1.19 and 1.04 fold larger than those of controls at 1 day, 4 days, 7days and 14 days after the injection, respectively (Table 1). Although values of controls between 1 day and 4 days, 1day and 7 days, 4 days and 7 days were not significant, the values of experiments were statistically significant (p<0.001) except for

between 1 day and 7 days (Fig. 5c).

Proliferating index analysis

Roughly 800 to 1,600 cells and 1,000 to 2,000 cells were observed within selected areas of controls and experiments, respectively. Mean values of proliferating index were significant differences between controls and experiments: 39.7±1% and 50.2±3% at 1 day, 43.2±1% and 68.9±4% at 4 days, 47.1±3% and 50.5±3% at 7 days, 43.0±3% and 50.4±2 at 14 days. Experimental values were 1.26, 1.59, 1.07 and 1.17 fold larger than those of

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controls at 1 day, 4 days, 7 days and 14 days after the injection, respectively (Table 1). Proliferating index of controls gradually increased and reached a peak at 7 days after the injection with a significant difference between each group, except for the 4-day group and the 14-day group, while the proliferating index value of the 4-day experiment was the highest one showing significant differences ($p<0.001$) comparing to values of other three experimental groups. There was no statistical difference between 1-day and 4-day, 1-day and 14-day, 7-day and 14-day experiments (Fig. 5d).

Discussion

Minute investigations on the development of mandibular condyle have been reported but there are no bFGF application data on mandibular condyle of growing mice *in vivo*. Although insulin-like growth factor (IGF) is an important factor in condyle development[8], it was reported that IGF local injection did not influence the 3-week-old rat of cartilage development[13]. In our study, it was suggested that total 0.3 μg administration of rhbFGF accelerated development of injected condyles, because all values calculated herein showed a statistically significant difference between experiments and controls. The dosage in our experiment was determined on the basis of the data reported by Shida *et al.* who injected from 1 to 20 μg bFGF into the knee joint of 4-week-Sprague-Dawley rats[12]. Their experiment showed that the joints injected with more than 5 μg of bFGF were larger than that in the control joints without bFGF injection and the effect of the injection was dose-dependent. Considering the weight of the 4-week-old Sprague-Dawley rats used in their experiment is about 50 g, an injection of 5 μg bFGF is equivalent to a 0.1 μg bFGF injection into a newborn mouse weighing 1 g.

Recombinant human bFGF works in low dose. The ED_{50} of bFGF for the mitogenic activity in bovine aortic endothelial cell is 0.3 ng/ml *in vitro*[14]. An experimental study of corneal wound healing[15] showed that doses of 0.1 to 10 ng/ml bFGF were effective. However, the half-life of rhFGF is extremely short and is estimated at 0.9 minutes for T1/2alpha and 7 minutes for T1/2beta in mice after intravenous administration of 10 μg/kg[16]. Colin and his coworkers[17] reported that intravenously administrated bFGF was rapidly accumulated in almost all solid organs within 5 minutes and more than 65% of FGF was retained in liver. Twenty four hours after administration, native bFGF had totally disappeared in liver. In the investigation of pericardial administration, 19% of bFGF delivered in cardiac tissue was present at 150 minutes after administration[18]. Although there is no detailed data on pharmacokinetics of bFGF subcutaneous administration, it was reported that radiolabelled bFGF after a single injection were remained in the knee joint only several hours[12]. Considering these investigations, it seems that subcutaneously injected bFGF remains for more than 2 hours and

is mostly cleared within 24 hours. The effect of a single injection of low dose bFGF was limited to condylar development. Therefore, we tried daily repetitive injections for three days in this examination. Although we have not the full answer to whether three time injections were appropriate, this investigation gave us interesting data as discussed below.

Histologically, control specimens showed that the thickness of mature and hyperplastic chondrocytes in the early stage (1 day and 4 days after injection) were thicker than those in the late stage (7 and 14 days after the injection). In other words, the erosive zone, which is the front of endochondral ossification, moved toward the top of the condyle. Morphometrically, both values of the thickness of proliferative zone and proliferating index reached their peaks at 7 days after the injection. Transition from active proliferation to thinning of mesenchymal cells and from thickening cartilage formation to active endochondral ossification is compatible with the normal development of mandibular condyle that has a capability for adaptive remodeling in response to external stimuli during or after natural growth[10,19]. Usually, lactation, weaning and mastication periods are from birth to approximately 1 week, around 3 weeks and approximately 3 weeks onward after birth. Current data was analyzed from 3 days to 17 days after birth; therefore, histological and morphometric changes seemed to be caused by the transition from lactation period to weaning period[20, 21].

Morphometric analysis clearly showed that the thickness of proliferative zone was significantly different between the controls and the experiments. The proliferation tendency of experimental condyles reached its peak at 4 days and decreased with time course. At this stage, the experimental value of proliferative zone thickness was about 1.6 fold higher than that of the control. On the other hand, the proliferation tendency of control condyles reached a peak at 7 days after the injection. This difference could be caused by stimulation of mesenchymal proliferation by bFGF administration. In order to evaluate thickening of fibrous and proliferative zones against vertical growth, we analyzed the ratio of proliferative zone thickness and the height of the condyle. This data also showed a same tendency to the proliferative zone thickness. The peak of the proliferating index of the experiment was also at 4 days after the injection, whereas that of the control was at 7 days. The highest control-experiment ratio of proliferation index value was nearly 1.6 at 4 days similar to that of proliferative zone thickness at 4 days. As above mentioned, mesenchymal cell thickening and cell proliferation status are intimately related to each other. Basic FGF inhibits matrix production and increases cell proliferation resulting in clustering of cells in intervertebral discs[6] and the proliferation of mandibular condylar chondrocytes [21]. Zheng *et al.* reported that bFGF-transfected human mesenchymal cells derived from bone marrow cells had a highly proliferative ability[22]. Taken together with our data, it was thought

that bFGF local injection resulted in mesenchymal cells proliferation, which was especially effective in the proliferative zone. However, its effect seems to be limited to less than a week.

As mentioned above, the half-life of rhFGF was extremely short[16] but the peak of cell proliferation reached at 4days after the final injection. This seems to be somewhat contradictory to the half-life of rhFGF. However, some growth factors synergistically act in cartilaginous development. Shi and his coworkers[23] demonstrated that bFGF increased both transcripts and proteins of bFGF and TGF-beta1 *in vitro*. They also showed that TGF beta1 significantly increased bFGF mRNA and production. The peak effect of bFGF production by TGF beta1 was at 4 days in contrast to at 12 hours by bFGF only. Thus synergic effects of growth factors after the final injection might prolong the acceleration of mesenchymal cell proliferation *in vivo*.

Interestingly, chondrocytes and hypertrophic chondrocytes of experiments increased in thickness at 4 and 7 days, and the distance from the condylar top to the edge of erosive zone of experiments was greater than that of controls. This feature may represent the delay of endochondral ossification. Administrated bFGF could possibly promote the differentiation of mesenchymal cells and pre-hypertrophic chondrocytes expressing collagen type II[24], but this factor inhibits terminal differentiation of chondrocyte[25] and matrix components in the mandibular condyles[26]. The mechanism of chondrocyte differentiation via exogenous bFGF is unknown in our experimental model. Previous study showed that bFGF reduced runt-related transcription factor 2 (Runx2)[27], which was closely related to upregulation of hypertrophic chondrocyte-specific Col10A1 gene[28]. There is a possibility that chondrocyte maturation is inhibited through suppression of Runx2 function. This might be the difference of the histological feature between the cartilaginous thicknesses of the control and experiment. However, smad3 or TGF-beta1 signaling also represses terminal hypertrophic differentiation of chondrocyte, and these are essential for maintaining articular cartilage[29,30] under the inhibition of TGF-beta signaling by negative regulator, such as an oncoprotein Ski[31]. The alteration of growth factor gene expressions after bFGF injection should be investigated to clarify the regulation of chondrocyte differentiation.

In conclusion, additional experiments on dosage, administration interval and frequency are necessary in order to obtain further effects of condylar development. However, our experiment demonstrated that locally administrated bFGF could accelerate mesenchymal cell proliferation of mandibular condyle in the lactation period after birth. This model seems to be useful for clarifying bFGF function against cartilaginous differentiation, condyle development and endochondral ossification of mandibular condyle *in vivo*.

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