Bone regeneration with systemic administration of lactoferrin in non-critical-sized rat calvarial bone defects

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Abstract: Using in vivo microfocus computed tomography (micro-CT) and tissue sections, we evaluated bone regeneration in non-critical-sized rat calvarial flat bone defects during systemic administration of lactoferrin (LF). Ten 11-week-old male Fischer rats were used. Non-critical-sized calvarial bone defects (diameter, 2.7 mm) were trephined into the dorsal parietal bone on both sides of the midsagittal suture, and a collagen sponge soaked in saline was placed on each side. LF was injected intraperitoneally every day, starting on the day of the operation (day 0; groups: control, 10 mg/kg LF, 100 mg/kg LF). Micro-CT imaging was performed repeatedly from 1 to 4 weeks after surgery. The defect sites were then removed, along with surrounding bone and soft tissues, and stained with hematoxylin and eosin. During weeks 1-4, micro-CT showed a significant difference in reossification ratio between the controls and the 100 mg/kg LF group. Histological analysis revealed that there were more osteoblast-like cells around the bony rim in the 100 mg/kg group than in the control group. In summary, micro-CT and histological analyses showed that systemic administration of LF accelerated bone regeneration in non-critical-sized rat calvarial bone defects. (J Oral Sci 55, 343-348, 2013)

Keywords: lactoferrin; bone regeneration; non-critical-sized rat calvarial flat bone defect.

Introduction
Lactoferrin (LF) is an 80-kDa iron-binding glycoprotein in the transferrin family of proteins (1). It is produced by many exocrine glands and is thus widely distributed in body fluids, including milk, saliva, tears, bile, and pancreatic fluid (2). LF is a pleiotropic factor with a wide range of biological functions. It is an iron-chelating agent and may contribute to antibacterial action (3). It also affects cell growth and differentiation (4), embryonic growth (5), endothelial cell adhesion (6), cytokine production (7), immune system regulation (8), and inflammatory response modulation (9). An in vitro study showed that LF potently activates osteoblast proliferation and differentiation.

Recent studies have shown that LF promotes bone growth (10,11). Local injection of LF in the calvariae of adult mice for 5 consecutive days increased bone formation and bone area as compared with controls (10). In addition, oral administration of LF to ovariectomized (OVX) rats for 3 months inhibited ovariectomy-induced loss of bone volume (BV) and mineral density (12). Moreover, bone formation was induced by bioresorbable collagen sponges loaded with growth factors, including
bone morphogenic proteins (13), platelet-derived growth factor (14), and basic fibroblast growth factors (15). In addition, microfocus computed tomography (micro-CT) and histological analysis showed that LF accelerated bone regeneration in non-critical-sized rat calvarial bone defects (16). In the present study, we examined the effect of systemic LF administration on bone regeneration in rat calvarial bone defects.

Materials and Methods

Animals

Ten 11-week-old male Fischer rats weighing 250-300g were used. The animals were housed in an experimental animal room (22°C, 55% humidity, 12/12 h light/dark cycle) and fed a standard laboratory diet and water. The Animal Experimentation Committee of the Nihon University School of Dentistry approved the present study (AP10D032-2).

Surgical procedure

After establishing general anesthesia with intraperitoneal (IP) sodium pentobarbital (30 mg/kg, Somnopentyl; Schering-Plough, Munich, Germany), the surgical area was shaved, the skin was washed with 70% ethanol, and 0.5 mL 2% lidocaine (Xylocaine; Astra-Zeneca, Osaka, Japan) was injected into the periosteum to control bleeding and provide additional local anesthesia.

A horseshoe-shaped skin incision was made over the head, the parietal area was exposed under aseptic conditions, and the periosteum was elevated to expose the bone. Non-critical-sized calvarial bone defects (diameter, 2.7 mm) were trephined into the dorsal bone on both sides of the midsagittal suture (Fig. 1a). Defects were created using a dental surgical drilling unit equipped with a trephine, which was cooled constantly with sterile saline. Then, the calvarial disk was carefully removed, to avoid tearing the dura. After thoroughly rinsing the area with physiological saline solution to wash out any bone fragments, an absorbable collagen sponge (Teruplug; Terumo Co., Tokyo, Japan) was placed (Fig. 1b). The skin was closed with 4-0 silk sutures (Ethicon, Somerville, NJ, USA). The day of surgery was designated as day 0.

LF administration

Rats were randomly assigned to three groups of 10 rats each and given the following treatments: control group (saline), 100 mg/kg LF (100 mg/kg body weight), and 10 mg/kg LF (10 mg/kg body weight). IP injection of LF or saline was done every day from day 0 to 4 weeks after the operation.

Imaging system

The R_mCT system (L9181S; Hamamatsu Photonics, Hamamatsu, Japan) has a microfocus X-ray tube with a focal point of 7 µm and an X-ray sensor with a 4-inch image intensifier. The X-ray source and image intensifier are connected by a basal plate, and the I-arm rotates on the vertical plane and is driven by a direct-drive motor. Rats were anesthetized with sodium pentobarbital and placed on the stage, and images of the areas of interest were captured. Repeated R_mCT imaging was performed from 1 to 4 weeks after surgery.

Micro-CT

The exposure parameters were 90 kV and 88 µA. The images were reconstructed on a personal computer using the I-View software (I-View Image Center, Tokyo, Japan). Using BV-measuring software (Kitasenjyu Radist Dental Clinic, I-View Image Center), voxel images were used to measure BV within the cylinder; gray values and numbers of voxels with corresponding gray values were calculated in regions of interest (ROIs; Fig. 2). Bone mineral density (BMD) phantoms of 200, 300, 400, 500, 600, 700, and 800 mg hydroxyapatite/cm³ with epoxy resin (Ratoc Engineering Co., Ltd., Tokyo, Japan) were

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**Fig. 1** (a) Non-critical-sized calvarial bone defects (diameter, 2.7 mm). (b) Absorbable collagen sponges soaked in saline were placed in the defects.

**Fig. 2** Observation area visualized by R_mCT.
scanned with the R_mCT under the same conditions used for the rats in this study. BMD was calculated using X-ray absorption values obtained from raw R_mCT data. The X-ray absorption threshold was determined to be 400 mg/cm$^3$. ROIs were defined on the bone defects, each of which had a diameter of 2.7 mm and a height of 3 mm. The amount of new bone growth in each ROI was then determined. BV was calculated by multiplying the number of pixels with $>$400 mg/cm$^3$ BMD by the volume of one pixel. BV in the ROIs was measured on day 0 and each week thereafter under the same conditions. Increase in BV, which is considered to indicate defect reossification, was then calculated by subtracting BV on day 0 from each subsequent value. In this manner, the defect reossification ratio was calculated every week.

Correlations with BMD were calculated using the pixel values of X-ray absorption from BMD phantoms of 200, 300, 400, 500, 600, 700, and 800 mg hydroxyapatite/cm$^3$. Bone mass (BM) was then calculated using the pixel values of X-ray absorption within ROIs, in combination with BMD correlations.

**Histological analyses**

Four weeks after surgery, the animals were killed by deep anesthesia using sodium pentobarbital (100 mg/kg IP; Somnopentyl). The skin was dissected, and the defect sites were removed, along with the surrounding bone and soft tissues. Next, the specimens were fixed in 10% formalin, after which they were decalcified with a formic acid-sodium citrate decalcification solution for 1 week and embedded in paraffin wax. Then, coronal sections (thickness, 5 µm) through the center of each circular defect were prepared and processed for hematoxylin and eosin staining. Histological examination was performed under a light microscope equipped with a morphometric system (manufacturer’s information), which was connected to a personal computer.

Defect closure was determined by measuring the distance between the defect margins and was expressed as a percentage of the width of the total defect (Fig. 3). New bone area was measured by counting the number of pixels representing all tissues within the boundaries of the newly formed bone. The mean numbers of osteoblast-like cells in all new bone areas were determined manually under a light microscope at ×200 magnification (Fig. 3).

**Serum collection**

Blood was collected from the tail vein 4 weeks after the operation. The blood was centrifuged and serum was collected. Serum specimens were stored at −80°C. Ca
Means and standard deviations of reossification ratios were calculated each week. The Mann-Whitney U-test was used to compare mean reossification ratios between groups. The significance level for statistical analysis was set at $P < 0.05$. Statistical analyses were performed using the SPSS software package (version 16.0J for Windows; SPSS Inc., Chicago, IL, USA).

**Results**

**Micro-CT**

Newly generated bone was observed as early as 2 weeks after surgery in the LF groups. Reossification developed by extensions of growth from the bony rims at the lateral sides of bone defects. Minimal new bone was observed in the control group (Figs. 4-6).

As compared with the control group, BV was significantly greater in the 100 mg/kg group at 1 week and later, and in the 10 mg/kg group at 2 weeks (Fig. 7).

**Histological analysis**

In the 100 mg/kg and 10 mg/kg groups, new bone formed from the margins towards the centers of bone defects. Osteoblast-like cells were observed around the bony rims of defects in the 100 mg/kg and 10 mg/kg groups. The absorbable collagen sponge structure was absorbed, and fibrous tissue, including osteoblasts and new bone, partially covered the LF sites (Figs. 8, 9). The control was filled with dense fibrous connective tissue; new bone formation near the defect rims was minimal (Fig. 10).

**Histomorphometric analysis**

Defect closure differed significantly between the 100 mg/kg group and control group (68.4% vs. 17.0%, respec-
The 100 mg/kg group had significantly larger areas of new bone as compared with control sites (P < 0.05; Table 1). There were more osteoblast-like cells in the 100 mg/kg and 10 mg/kg groups than in the control group (Table 2).

Serum collection
There was no significant difference in serum Ca concentration (Fig. 11). ALP increased with LF dose, but the differences between groups were not statistically significant (Fig. 12).

Discussion
Continuous administration of LF for 5 consecutive days tended to dose-dependently increase new bone formation in the calvariae of adult mice. In a previous study, a 4 mg dose induced changes that were 4-fold those observed in control animals. We previously applied LF-permeated absorbable collagen sponges directly to bone defects and found that this was effective in inducing bone regeneration (16). In the present study we found that systemic administration of LF enhanced bone regeneration in non-critical-sized rat bone defects and identified the LF concentration that inhibited bone resorption in OVX rats (12). This is the first report that systemic administration of LF promotes bone regeneration in bone defects.

Dietary administration of LF improved BMD in an OVX rat model (12,17), which suggests that LF-induced improvement in bone metabolism is due to a direct local effect of LF on bone. However, it is uncertain whether dietary LF reaches systemic circulation. We therefore used IP injection in this study. Guo et al. (12) reported that serum Ca concentration was inversely associated with LF dose and believed this explained the decrease in osteoporosis in OVX rats. In our non-OVX rats, systemic LF administration did not affect serum Ca level. Hou et al. (17) reported that serum ALP level increased in relation to LF dose. We also found that serum ALP levels tended to increase with LF dose, but the differences between groups were not statistically significant.

As compared with the control, production of osteoblast-like cells at wound sites was more than doubled in rats given LF. A previous study using primary rat osteoblasts cultured for 3 weeks found that bone nodule formation (a process that involves bone matrix deposition and mineralization by differentiated osteoblasts) depended on LF dosage and that both the range of mineralized bone formation and number of nodules increased. LF was strongly related to proliferation of primary osteoblast cells and osteoblastic cell lines and increased osteoblast differentiation (18). The mitogen stimulatory action of LF on osteoblast cells is mainly mediated by low-density lipoprotein receptor-related protein 1 (19).
According to Cornish et al. (10) LF has an anabolic action on osteoblasts and an inhibitory effect on osteoclast cells in vitro, which suggests that it positively affects BV in vivo. In a previous study we applied LF directly to bone defects (16) and found that BV was significantly greater than in this study. Local application of LF may thus act directly on osteoblast cells in bone defects.

Mountzias et al. (20) reported that control of inflammatory reactions promoted bone regeneration. Since inflammation is associated with a primary reduction in BV, an anti-inflammatory effect is useful for bone regeneration. It has been reported that LF has anti-inflammatory effect. Moreover, LF has a known clinical safety profile and is not carcinogenic or toxic (21,22).

Several studies have shown that growth factors promote bone regeneration in vivo (13-16). We believe that LF is more cost effective (11) and safer than other growth factors. In addition, LF can be administered in bovine milk, along with other dietary constituents and supplements. Thus, it is also more practical than other growth factors.

References