Bone regeneration by lactoferrin released from gelatin hydrogel

RYOHEI TAKAOKA[1,2], YOSHIAKI HIKASA[2], KENTARO HAYASHI[1] and YASUHIKO TABATA[1]*

[1]Institute for Frontier Medical Sciences, Kyoto University, 53 Kawara-cho Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

[2]Department of Veterinary Internal Medicine, Faculty of Agriculture, Tottori University, Minami 4-101, Koyama-cho, Tottori 680-8553, Japan

*To whom correspondence should be addressed.

Tel: (81-75)751-4121, Fax: (81-75)751-4646

E-mail: yasuhiko@frontier.kyoto-u.ac.jp

Running title; Bone regeneration by LF release

Abstract

The objective of this study is to evaluate the potential of lactoferrin (LF), an iron-binding glycoprotein, to induce bone regeneration. A biodegradable hydrogel of gelatin was prepared to allow the LF to release in vivo in a sustained fashion. When subcutaneously implanted into the back of mice, the gelatin hydrogel incorporating LF showed a longer time period of LF retention at the site implanted than that of LF solution infection. An in vitro culture experiment of 3T3E1 cells (mouse derived osteoblasts) revealed that the cells were proliferated by the repeated addition of LF to a significantly great extent compared with the single addition of LF at the same dose. Following the implantation of gelatin hydrogels incorporating LF into a skull bone defect of rats, significantly stronger bone regeneration at the defect was observed than LF-free or -low. It is concluded that the sustained release with the gelatin hydrogels enables LF to enhance the in vivo activity of bone regeneration.

Introduction

Lactoferrin (LF) is an iron-binding glycoprotein of transferring family. It is present in breast

milk, especially colostrum (6-8 mg/ml) and in the secondary granules of neutrophils. It has been reported that LF has several biological activities, such as the enhancement of cell proliferation and differentiation [1, 2], and endothelial cells adhesion [3], anti-tumor [4], and the modulation of inflammatory responses [5]. In addition, it modifies the proliferation and differentiation of osteoblasts [1, 6] and can inhibit the cell apotosis [7]. On the other hand, LF increases the calcification of extracellular matrix by human osteoblast-like cells [8], which experimentally a great potential of LF for bone regeneration.

It is widely recognized that growth factors play an important role in tissue regeneration at different stages of cell proliferation and differentiation [9]. However, successful tissue regeneration by the growth factor has not been always achieved. One of the reasons is that the in vivo half-life period of growth factor is too short to expect the biological activities. It is necessary for their enhanced in vivo activity to develop the drug delivery system (DDS) of growth factor. For example, it has been reported that the sustained release of bone morphogenetic protein-2 (BMP-2) enhances the activity to induce bone formation whereas the

solution was not effective in vivo [10, 11].

Various materials have been investigated as the material for sustained release of growth factors [12]. Among them, gelatin is being used for the material of various growth factors release, such as basic fibroblast growth factor (bFGF) [13], bone morphogenetic protein 2 (BMP-2) [14], transform growth factor (TGF) β-1 [15] and vascular endothelial growth factor (VEGF) [16]. Gelatin has been widely used for pharmaceutical and medical applications to demonstrate the biocompatible and biodegradable natures on the basis of the long-term clinical trials. Stallmann et al. reported that the continuous release of human LF from calcium phosphate bone substitutes [17], was effective in enhancing bone regeneration.

The objective of this study is to evaluate the potential of LF to induce bone regeneration when LF is applied in a sustained release fashion. A biodegradable gelatin hydrogel was prepared to allow LF to release in a sustained manner. After applied to the bone defect of rat skulls, the bone regeneration by the hydrogel incorporating LF was assessed. We examine the in vitro proliferation of cells by addition of LF in a repeated fashion and compared with that of

cells cultured LF added one time.

Materials and Methods

Materials

A gelatin sample with an isoelectric point of 5.0 was kindly supplied by Nitta Gelatin (Osaka, Japan). Bovine LF (WAKO Pure Chemical Industries, Osaka, Japan, Lot No ALR2632.), glutaraldehyde (GA), glycine, and other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan) and used without further purification.

Preparation of gelatin hydrogels

Gelatin hydrogels were prepared by the chemical cross-linking of gelatin with GA. Briefly, 4.29 wt% aqueous solution of gelatin (70 ml) was mixed at 5,000 rpm at 37 °C for 3 min with a homogenizer (ED-12, Nihonseiki Co., Tokyo, Japan). After the addition of 2.17 wt% GA aqueous solution (30 ml), the mixed solution was agitated for 15 second by the homogenizer.

The resulting solution was cast into a polypropylene dish of 138×138 cm^2 and 5 mm depth, followed by leaving at 4 °C for 12 hr for gelatin cross-linking. Then, the cross-linked gelatin hydrogels were placed into 100 mM of aqueous glycine solution at 37 °C for 1 hr to block the residual aldehyde groups of GA. Following complete washing with double distilled water (DDW), the hydrogels were freeze-dried and cut into the disc in 8 mm diameter and 2 mm thickness for the following experiments.

Sustained release test of LF from gelatin hydrogels

LF was radioiodinated according to the method of Greenwood et al.[18]. Then, 20 µl of aqueous solution containing ^{125}I-labeled LF was dropped onto a freeze-dried gelatin (2 mg), and then left 37 ℃ for 1 hr to obtain the gelatin sponge incorporating ^{125}I-labeled LF. Since the volume of LF solution added was much smaller than that theoretically impregnated into the sponge, 100 % of LF added could be completely incorporated into the hydrogel. The gelatin hydrogel incorporating ^{125}I-labeled LF was implanted into the back subcutis of 6-week-old female DDY mice (Shimizu Laboratory Animal Supply Co., Ltd., Kyoto, Japan) at the central

position 15 mm away from the tail root. As a control, 20 μl of aqueous solution of ^{125}I-labeled LF at the same dose was subcutaneously injected into the mouse back. Each experimental group was composed of three mice. At different time intervals, the mice were sacrificed, and the back skin (3×5 cm^2) around the sample implanted or injected site of LF was cut out and the corresponding facia was thoroughly wiped off with a filter paper to absorb the ^{125}I-labeled LF remaining. The radioactivity of remaining gelatin hydrogel, the skin piece, and the filter paper was measured on the gamma counter (ARC-301B, Aloka Co., Ltd., Japan). The percentage of remaining radioactivity was expressed as the radioactivity ratio of test samples to the original hydrogel or aqueous solution containing ^{125}I-labeled LF.

Cell culture experiment with LF

MC3T3-E1 cells (mouse derived osteoblasts) were seeded into each well of 24-well multi dish culture plate (Corning Inc., Corning, NY) at a cell density of 2×10^4 cells / well and cultured in 500 μl of culture MEM medium with 10 wt% fetal bovine serum (FBS) for one day (day 0). After the incubation for one day, each well was washed twice with the phosphate-buffered

saline solution (PBS, pH 7.4) twice. Then, the cells were cultured in FBS-free medium for 3 days. Fresh FBS-free medium (500 μl/well) was changed every day, LF was added into the medium in one-time or repeated time fashion. For the group of one-time, single addition, the LF solution was added to each well at total concentrations from 0.15 to 1500 μg/ml on day 1. On day 2 and 3, 500 μl of PBS was added to each well. For the group of repeated addition, the LF solution was added 3 times to each well at the total concentrations from 0.05 to 500 μg/ml every day for 3 days. As a control group, MC3T3-E1 cells were incubated in the medium containing 10 wt% FBS for 3 days, while the medium was changed every day by the PBS addition at same amount of other groups.

After the 3-day culture, the cells were washed twice with PBS. The culture medium (500 μl) was added into each well. The cell proliferation was evaluated with MTT assay kit (Nacalai tesque Inc., Kyoto, Japan). Briefly, to each well, 50 μl of solution (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt; WST-8) was added followed by incubation for futher 2 hr. The absorbance of cell supernatants was measured on a VERSAmax microplate reader (Molecular Devices,

Sunnyvale, CA, USA) at 450 nm. The number ratio of cell proliferated was expressed as 1.0 for the group where cells were cultured in the medium containing 10 wt% FBS.

Bone regeneration assay of gelatin hydrogels incorporating LF

LF solution at concentrations of 0, 1, 10, and 100 mg/ml (300 μl), was dropped onto the disc of gelatin hydrogel freeze-dried, followed by leaving at 4 ℃ for overnight in dark to obtain the gelatin sponge incorporating 0, 0.3, 3, and 30 mg of LF. Fisher 344 rats (14-week-old, Shimizu Laboratory Animal Supply Co., Ltd., Kyoto, Japan) were used divided into 4 groups (3 rats / group). Under anesthesia with a pentobarbital (40 mg/kg), a flap was raised and a bone defect of 8-mm diameter was created with a bone trephine bur. Then, the hydrogels incorporating LF were applied to the defect, and then the flap was repositioned and sutured. The rats were sacrificed 8 weeks after the hydrogel application. The calvaria were dissected out and examined with soft X-ray radiography (Hitex-100, Hitachi, Tokyo, Japan) at 20 kVP and 2.0 mA for 200 sec. X-ray radiographs around the bone defect (8 × 8 mm square) were mechanically changed to the binary images on a soft ware (Photoshop, Adobe Systems

Incorporated, San Jose, CA, USA) at the region of interest of 8 × 8 mm square. Then, 3 binary images for each experimental group were analyzed to calculate the average percentage of ossified area ± the standard error.

Statistic analysis

Statistical analysis was performed by the Tukey-Kramer method, and p value less than 0.05 was considered significant.

Results

Release profile of LF from gelatin hydrogels incorporating LF

Figure 1 shows the release profile of LF from gelatin hydrogels incorporating with ^{125}I-labeled LF after subcutaneous implantation. The remaining radioactivity of ^{125}I-labeled LF solution injected subcutaneously was 2.96 and 0.82 % on Day 1 and 3, respectively. On the contrary, the remaining radioactivity of ^{125}I-labeled LF in the hydrogel incorporated form was 10.14 and 5.26 % on Day 1 and 3. The remaining radioactivity of LF for a long time period was

observed by the sustained release with the hydrogel.

In vitro cells proliferation

Figure 2 shows the number of cells proliferated in the presence of LF in the one-time or repeated additional fashion. With an increase of LF concentration, the cell proliferation was promoted. At higher concentrations of LF, the number of cells proliferated was significantly higher for the repeated additional group than that of single additional group.

In vivo bone regeneration by gelatin hydrogels incorporating LF

Figure 3 shows the soft X-ray radiography pictures of calvarial defect after implantation of gelatin hydrogels incorporating various amounts of LF. Implantation of LF-free gelatin hydrogels did not induce bone regeneration. On the other hands, bone regeneration at the defect site was observed by the implantation of gelatin hydrogels incorporating 30 mg of LF.

Discussion

The present study demonstrates that the sustained release with the gelatin hydrogel enabled LF to enhance the potential of bone regeneration. Stallman et al. reported that the continuous-release of human LF from calcium phosphate bone substitutes was effective in enhancing bone regeneration [17]. The release technology is necessary to enhance LF-induced bone regeneration.

It is well recognized in the polymer science that a positively or negatively charged polyelectrolyte ionically interacts with the oppositely charged one. Thus, it is highly possible that the LF molecule of a positive charge is not released from the acidic gelatin hydrogel (negative charge) unless the hydrogel is degraded to generate water-soluble fragments. On the other hand, LF will be diffused out of the basic gelatin hydrogel of positive charge since there is no electrostatic interaction between the LF and gelatin molecules. The initial release from the acidic gelatin hydrogel can be explained in terms of this LF diffusion. Because the present LF impregnation condition was not sufficient to complete poly-ion complexation between the LF and the acidic gelatin molecules, the uncomplexed LF would be diffused out

initially. There have been reported on many carriers for the controlled release of proteins[10, 16, 19, 20]. The profile of LF release from other carriers is under investigation at present to compare that of gelatin hydrogel.

We investigated the effect of LF additional fashion on the proliferation of cells. Repeated addition of LF was more effective in enhancing the cell proliferation than the single addition. The enhanced extent became high as the LF concentration increased. Cornish et al. investigated that the effect of bovine LF on the proliferation of human osteoblasts to demonstrate the promoted proliferation of primary or cell line cultures of human or rat osteoblast-like cells in the dose-dependent manner (1-100 μg/ml). It is reported that in the healthy body, the serum level of LF ranges from 2 μg/ml to 7 μg/ml [21]. On the other hand, Lorget et al. have reported that bovine LF inhibited the in vitro bone resorption (200 μg/ml) in the in vitro culture of rabbit bone cells. Therefore, the concentration of LF to affect the cell behavior in vitro should be examined considering the type of cells. In this study, we did not check the time course of lactoferrin concentration in culture medium and around bone defect.

These points should be checked in order to confirm the effective concentration of lactoferrin is released from the hydrogel.

It is apparent in Figure 2 that the repeated addition of LF effectively enhanced the in vitro proliferation of cells. This is just an in vitro experiment, but this clearly indicates that a continuous supply of LF to the cell culture system was effective in the enhancement of cells proliferation. Bone regeneration could be detected in a skull bone regeneration model 8 weeks after implantation. On the other hand, ^{125}I-labelled lactoferrin was not detected in the tissue around implanted hydrogel on 14 days after implantation. This time difference is often observed in the case of protein-induced bone regeneration. The protein is locally released for 14 days to enhance the number of key cells and activate their activity. Then, the cells initiate to work on the regeneration of bone tissues. It is possible that it needs some time period to achieve the cell-based bone regeneration. It is well known that LF interacts with the receptors of cells to exert their biological functions. There is the cell surface receptor of LF for various types of cells, such as T cells [22], monocytes [23], liver cells [24], intestinal cells [25], platelets

[26], and kidney, thyroid or parathyroid glands [27]. However, we did not check which cells were key cell affected by lactoferrin.

There are two important advantages to use gelatin hydrogels over the LF solution upon applying to the defect site. One of the advantages is the sustained release of LF which can be achieved with the gelatin hydrogel. The other is that the gelatin hydrogel could maintain the LF concentration at the bone defect site. The LF molecule of positive charge, once ionically complexed with the acidic gelatin of negative charge, is not released from the acidic gelatin hydrogel unless the hydrogel is degraded to generate water-soluble fragments.

As one control experiment, the repeated addition of LF was effective in the enhancement of cell proliferation in vitro. This indicates that continuous release enables LF to induce the biological activity. Taken together, we can say with certainly that the sustained release of LF promotes LF-based the ability of bone regeneration.

In vivo experiments (Figure 3) revealed that the gelatin hydrogel functioned well to increase the number of key cells, resulting in cell-based bone regeneration. Some papers demonstrate

that bone regeneration was promoted by growth factors, especially bone morphogenetic proteins (BMPs) [14, 19, 28] and basic fibroblast growth factor (bFGF) [29]. We think that comparing with other growth factors, an important advantage to use LF is the low cost compared with other growth factors. Smith et al. reported that bone regeneration in a rabbit calvarial was induced by 288 μg/defect of BMP-2 with a collagen sponge [28]. Yamamoto et al. succeed in the dose reduction of BMP-2 dose (17 μg/defect) induce bone regeneration by using the gelatin hydrogel of release carrier [14]. Tabata et al. demonstrate bone regeneration in a rat calvarial defect by gelatin hydrogel incorporating 100 μg of bFGF [29]. Although it is reported that the dual release of growth factors could reduce their dose requested to induce bone regeneration [30]. However, from the view point of cost, it is practically difficult to say that the growth factors are available for therapy. In this study, we use a 30 ㎎ of LF for bone regeneration in the rat calvarial defect. Mountziaris PM et al reported on the modulation of the inflammatory response for enhanced bone tissue regeneration [31]. An anti-inflammatory property is useful for bone regeneration since inflammation is mainly associated with a loss of bone mass. LF is reported to have an anti-inflammatory activity[5]. On the contrary, this

property is not observed for BMPs and bFGF. It is highly conceivable that LF has an advantage over other growth factors in terms of anti-inflammation.

This study experimentally confirmed that the combination of LF and gelatin hydrogel was useful to induce bone regeneration. However, it is difficult to make a direct comparison in the bone regeneration between this and other papers because there are some differences in the animal species, the defect model, and the speed and degree of bone regeneration. In spite of this, we believe that the cost-benefit performance of LF is very practical compared with other growth factors which are widely used for the bone regeneration.

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Figure legend

Figure 1

Time profiles of radioactivity remaining after the subcutaneous injection of ^{125}I-labeled LF solution (○) and implantation of gelatin hydrogel incorporating with ^{125}I-labeled LF (●).

Figure 2

Time profiles of MC3T3-E1 cells proliferation in the presence of LF in the single (□) and 3-times addition of LF (■)

†, $p<0.05$; significant against the number of cells cultured in the LF-free medium

††, $p<0.05$; significant against the number of cells cultured on the single addition of LF into medium

Figure 3

Soft X-ray radiographies of rat calvarial defect 8 weeks after implantation of gelatin

hydrogels incorporating 0, 0.3, 3, and 30 mg LF. The lower stand value indicates the average percentage of area ossified in 8 × 8 mm square around bone defect. No statistical significant was observed between LF 0 and 0.3 or 3 mg group.

✻, $p<0.05$; significant against the percentage of ossified area in square around defect implanted with gelatin hydrogels incorporating 0 mg of LF.

Figure 1

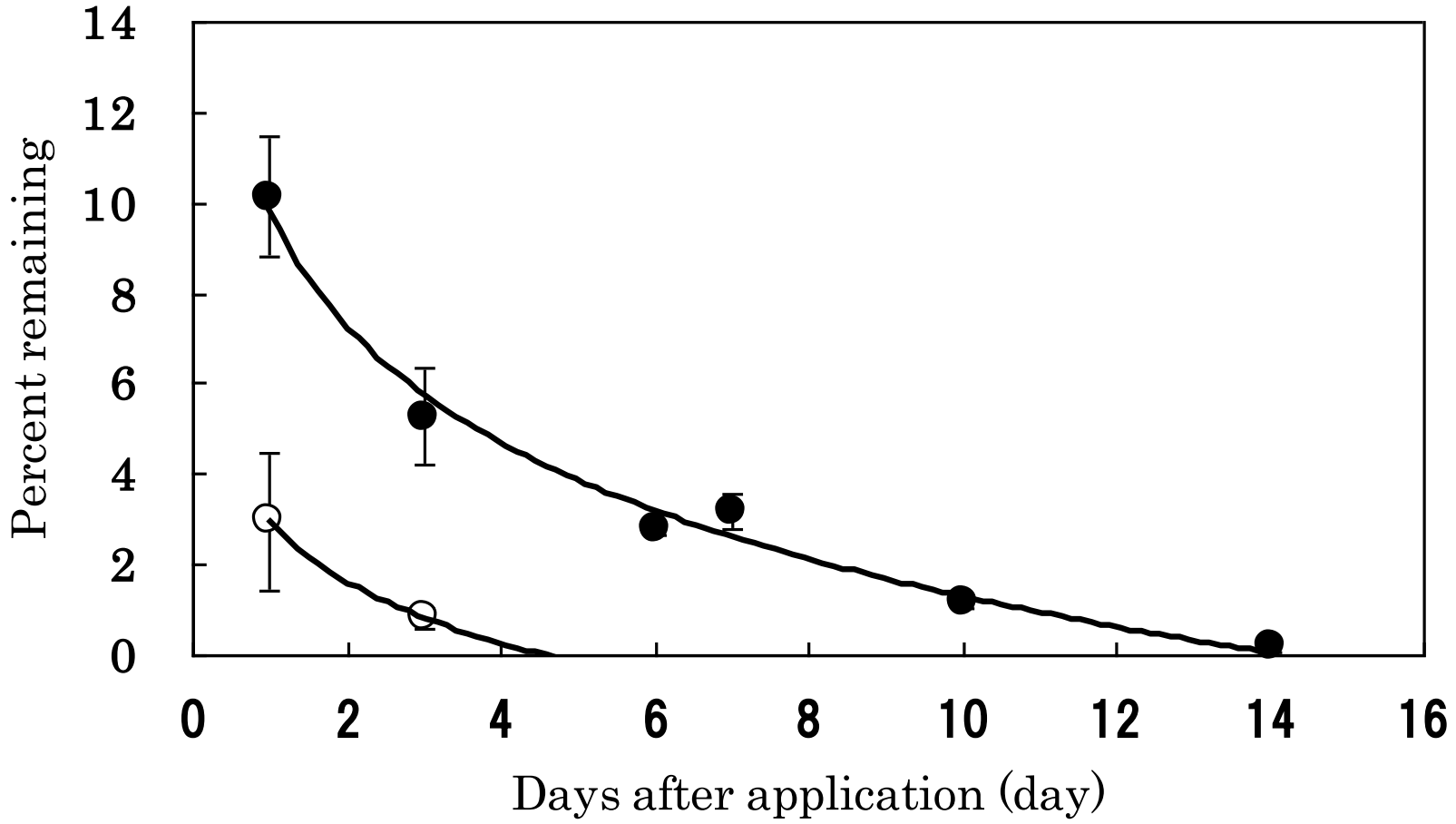

Percent remaining
14
12
10
8
6
4
2
0
0
2
4
6
8
10
12
14
16
Days after application (day)

Figure 2

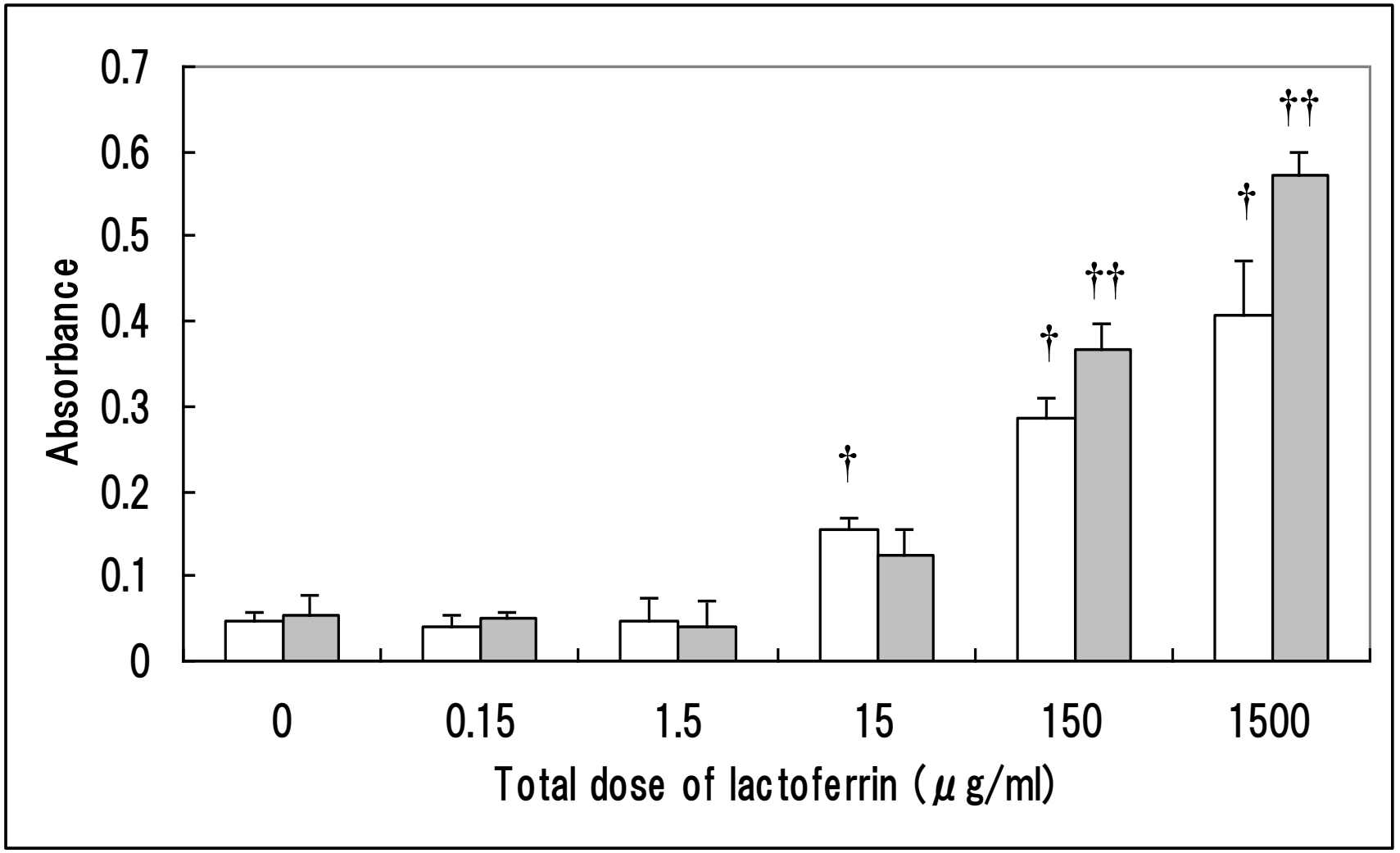
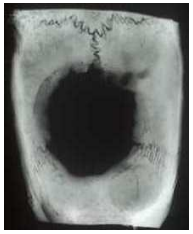
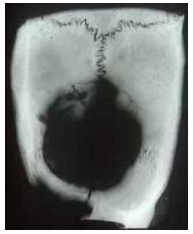
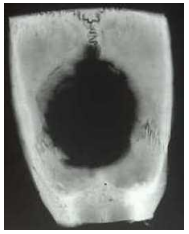
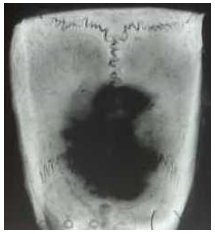
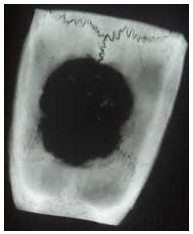
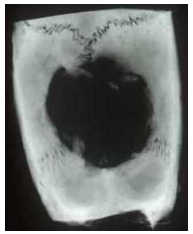
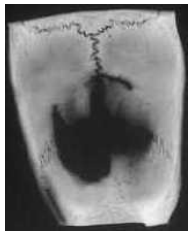
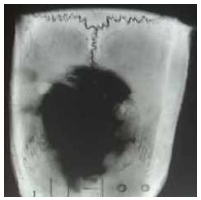
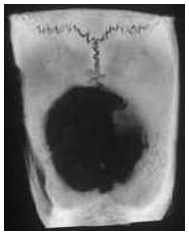
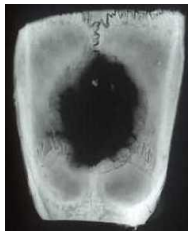
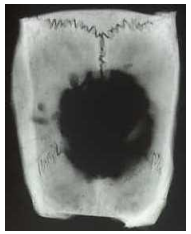
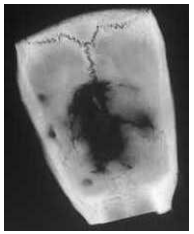

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance
†
†
††
†
††
0
0.15
1.5
15
150
1500
Total dose of lactoferrin (μg/ml)

Figure 3

Amount of LF incorporated (mg)

0	0.3	3	30
			
			
			
25.0±5.4	34.1±7.7	34.7±6.0	47.9±8.8*