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Bone regeneration by basic fibroblast growth factor complexed with biodegradable hydrogels

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Abstract

The objective of this study is to enhance the bone induction activity of basic fibroblast growth factor (bFGF) for reconstruction of skull bone defects which has been clinically recognized as almost impossible. For this purpose, we prepared biodegradable hydrogels from gelatin with an isoelectric point of 4.9 which is capable of polyionic complexing with basic bFGF. When implanted in rabbit skull defects of 6 mm in diameter (6 defects per experimental group), the gelatin hydrogels incorporating 100 μg of bFGF promoted bone regeneration at the defect in marked contrast to free bFGF of the same dose, finally closing the bone defects after 12 weeks of implantation as is apparent from histological examination. In dual energy X-ray absorptometry analysis, the bone mineral density at the skull defects enhanced by the hydrogels was significantly higher than that by free bFGF at doses ranging from 2 to 200 μg/defect ($P < 0.05$). The extent of bone regeneration induced by gelatin hydrogels incorporating 100 μg of bFGF increased with a decrease in their water content. Histological examination indicated that more slowly degrading hydrogels of lower water content prolonged the retention period of osteoblasts in the bone defects. This led to enhanced bone regeneration compared with faster degrading hydrogels of higher water content. It was concluded that this biodegradable hydrogel system was a promising surgical tool to assist self-reconstruction of the skull bone.

Keywords: bFGF; Gelatin; Bone regeneration; Polyion complex; Hydrogel

1. Introduction

Gene technology has made possible the mass production of bioactive proteins, but the half-life period of proteins in the body is generally too short to effectively exert the biological activity when they are injected in the free form. One possible method to circumvent this problem and enhance the in vivo efficacy is to release the proteins at the site of action over a long period of time. It is well recognized that a bFGF molecule has a high binding affinity for acidic polysaccharides in the extracellular matrix, e.g., heparin and heparan sulfate, probably for protection of bFGF from denaturation and enzymatic degradation in vivo [1, 2]. Thus, based on this inherent affinity of bFGF for acidic substances, we have prepared a biodegradable hydrogel composed of alkaline-processed 'acidic' gelatin with an isoelectric point (pI) of 4.9 which can ionically interact with bFGF. The gelatin hydrogel was degraded in vivo and the degradability could be controlled by altering the extent of gelatin crosslinking. The bFGF ionically complexed with the acidic gelatin was not released from the hydrogels under the in vitro non-degradation conditions. It is possible that the hydrogels achieved the sustained release of biologically active bFGF as a result of hydrogel degradation, resulting in significantly enhanced angiogenic effect in marked contrast to free bFGF [3].

Thompson et al. reported that the release of acidic FGF from gelatin was effective in enhancing the neovascularization activity in vivo [4]. In addition to the angiogenic effect, it has been demonstrated that bFGF

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stimulates proliferation of osteoblastic cells [5, 6] and enhances bone formation in the long bone of rats when injected intravenously in the solution form [7–10]. However, little research on the combination of bFGF with its carrier has been reported, although there is one report which described that a mixture of demineralized bone matrix and bFGF was effective in enhancing the bone formation in rat tibia [11]. These findings strongly suggest that if it is possible for bFGF to be localized and released at the site to be acted on for an extended time period, efficient bone formation will be achieved.

The present study was undertaken to evaluate the feasibility of our gelatin hydrogel system to enhance bFGF-induced osteogenetic effect. Since the hydrogel system is a suitable for the localization and sustained release of bFGF, it is highly expected for it to promote bone regeneration. bFGF-incorporating gelatin hydrogels were applied to the skull defects of rabbits to evaluate their potent efficacy as the material to promote bone regeneration at the skull defects which is known to heal much more slowly than long bone defects [12]. To our knowledge, any biological action of bFGF on skull bones has not yet been investigated. The skull bone regeneration induced by the bFGF-incorporating gelatin hydrogels was compared with that induced by free bFGF and evaluated in terms of their bFGF dose and degradation rate. We also describe the time course of the number of osteoblasts recruited in the skull defects during the process of bone regeneration.

2. Materials and methods

2.1. Materials

Gelatin with a pI of 4.9 was prepared by the alkaline treatment of bovine bone collagen with $Ca(OH)_2$ (Nitta Gelatine Co., Osaka, Japan). It was named as acidic gelatin due to the acidic feature since the amide groups of collagen were converted to carboxyl groups through the alkaline process. The weight-average molecular weight of the acidic gelatin was 99,000 when measured by gel filtration chromatography relative to standard poly(ethylene glycol) samples. Human recombinant bFGF with the pI of 9.6 was kindly supplied from Kaken Pharmaceutical Co., Tokyo, Japan. Other chemicals were purchased from Wako Pure Chemical Ltd., Kyoto, Japan and used without further purification.

2.2. Preparation of bFGF-incorporating gelatin hydrogels

Gelatin in 10 wt% aqueous solution was chemically crosslinked with various amounts of glutaraldehyde at 4°C to prepare hydrogels with different extents of crosslinking. Briefly, 4.5 ml of an aqueous gelatin solution containing glutaraldehyde at the final concentrations of 2.5, 5.0, 7.5, 20.0, 30.0 and 100.0 mM was cast into a Teflon mold ($5 \times 5\ cm^2$, 1.8 mm depth). The crosslinking reaction was allowed to proceed for 24 h at 4°C and the crosslinked hydrogel sheets were immersed in 50 mM glycine aqueous solution at 37°C for 1 h to block residual aldehyde groups of glutaraldehyde. Following punching out of the resulting hydrogel sheets to form into disc (6 mm in diameter), the hydrogel discs were rinsed by double-distilled water (DDW), 100% ethanol and autoclaved DDW to obtain the sterilized hydrogels. The sterilized hydrogels were aseptically freeze-dried. No big change in hydrogel shape was observed before and after freeze drying. Impregnation of bFGF into the freeze-dried hydrogels was carried out by use of phosphate-buffered saline solution (PBS, pH 7.4) containing 2, 10, 20, 50, 100 and 200 μg of bFGF. bFGF-free, empty gelatin hydrogels were prepared similarly except for using bFGF-free PBS. 30 μl of PBS with or without bFGF was impregnated into the dried hydrogels during the swelling process, irrespective of the water content of hydrogels, because the volume of the buffer was much less than that theoretically impregnated into each hydrogel. Fig. 1 shows the light microscopic photograph of bFGF-incorporating gelatin hydrogels and freeze-dried hydrogels before bFGF impregnation. The pore of freeze-dried hydrogels ranged from 20 to 60 μm. The size of bFGF-incorporating gelatin hydrogel discs obtained was 6 mm in diameter and 1.8 mm thick, irrespective of their bFGF loading and water content. The bFGF loading of the hydrogel was indicated as the amount of bFGF added in hydrogel swelling. The swollen hydrogels were used for the following experiments.

As one method to evaluate the crosslinking extent of gelatin hydrogels, their water content was measured. The hydrogel weight before and after swelling at 37°C in PBS was determined to calculate the water content, which was defined by the weight percentage of water in the wet hydrogel [3]. Gelatin hydrogels with the water contents of 98, 95, 92 and 85% were used for the following animal experiments. All experimental processes were done under sterile conditions.

2.3. Animal experiment

Japanese white rabbits weighing 2.0–2.5 kg were anesthetized by the intravenous administration of Nembutal® injection (Dainippon Pharamaceutical Co. Ltd., Osaka, Japan) which contains 50 mg of sodium pentobarbital, 0.4 ml of propylene glycol and 0.105 ml of ethanol per 1 ml of the solution ($0.6\ ml\ kg^{-1}$ body weight). In addition, local analgesia was performed by subcutaneous administration of 1% lidocaine solution into the subcutaneous tissue of rabbit heads (4 ml/head). The skin of rabbit heads was cut to expose their skull bone. After incision of the pericranium, the 2 skull defects of 6 mm in diameter per rabbit were made symmetrically

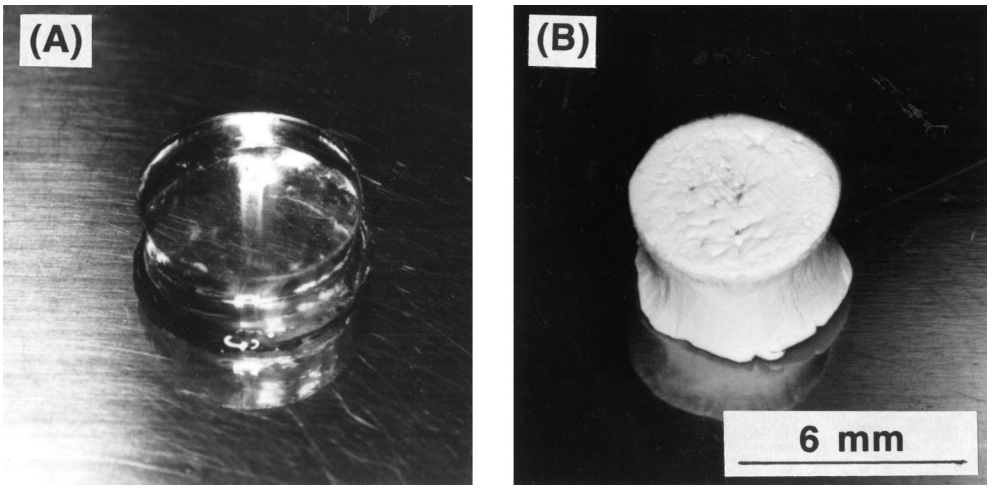

Fig. 1. A light microscopic photograph of bFGF-incorporating gelatin hydrogel with a water content of 85% (A) and dried gelatin hydrogel (B).

4 mm far from the sagittal suture at the parietal bone using a microdrill without injuring the underlying dura mater. All these operative procedures were performed by use of a surgical microscope. Institutional guidelines regarding animal experimentation were followed. Gelatin hydrogels incorporating different amounts of bFGF and bFGF-free gelatin hydrogels were placed to the rabbit skull defect. As controls, 30 μl of PBS with or without bFGF was applied to the skull defect. Six defects were randomly selected from the right or left defect of different rabbit skulls and treated with bFGF-incorporating gelatin hydrogels and other agents. Each experimental group composed of 6 defects from 6 different rabbits. Preliminary experiments demonstrated that bFGF treatment for one defect did not affect bone response to the counterpart defect, irrespective of the dosage form. The pericranium and skin were carefully sutured with 4-0 nylon monofilaments.

The animals were sacrificed by intravenous administration of sodium pentobarbital at an over-dose 4 days and 1, 2, 4, 8 and 12 weeks after treatment. The skull bone was drilled out together with the defects and fixed in 10 wt% formaldehyde solution in PBS for 4 days to assess the bone regeneration.

2.4. Assessment of bone formation and cell recruitment

Bone regeneration at the skull defect was assessed by Dual Energy X-ray Absorptometry (DEXA) and histological examinations. The bone mineral density (BMD) of each defect was measured by DEXA utilizing a bone mineral analyzer (Dichroma Scan 600, Aloka Co., Tokyo, Japan) at 4 days and 1, 2, 4, 8 and 12 weeks after treatment with or without bFGF. The instrument was calibrated with a phantom of known mineral content. Each scan was performed at a speed of 20 mm s^{-1} and the scanning length was 1 mm. DEXA measurement was performed for 6 defects per each experimental group and the region of interest (ROI) for each defect was 5×5 mm^2.

Bone specimens were demineralized in 10 wt% EDTA solution at 4°C for 3 days, embedded in paraffin and sectioned at 10 μm in thickness. The sections were prepared to cut as near as the center of the skull defect and stained with hematoxylin–eosin (HE) at 4 days and 1, 2, 4, 8 and 12 weeks post-implantation. The histological sections were viewed at a high magnification of 400× by a light microscope to count the number of osteoblasts in an active form according to the method reported by Nakamura et al. [10]. Measurements were performed at four squared-shaped areas (0.6×0.6 mm) including the edge of bone defects per one histological section. The area was randomly selected along the edge of bone newly formed around the margin of the defect hole. Six different sections were measured for each experimental group. The number of osteoblasts, being characterized by basophilic cuboided cytoplasm locating adjacent to the bone surface [13], was counted in each area. The bone edge perimeter in each area was measured using an image analyzer (SP-1000, Olympus, Tokyo, Japan) under a light microscope. The resulting cell number per perimeter was calculated from the data of 16 independent areas.

2.5. Statistical analysis

All the data were analyzed by Students' t -test to assess statistical significance between experimental groups. Experimental results were expressed as means ± standard error.

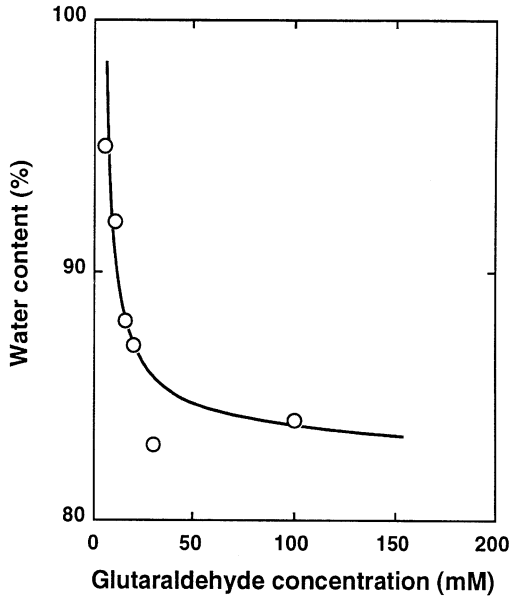

Fig. 2. The influence of glutaraldehyde concentration on the water content of gelatin hydrogels prepared at the gelatin concentration of 10 wt%, reaction temperature of 4°C and reaction time of 24 h.

3. Results

3.1. Characterization of bFGF-incorporating gelatin hydrogels

Figure 2 shows the relationship between the water content of gelatin hydrogels and the concentration of glutaraldehyde used in the hydrogel preparation. It is apparent that the water content of gelatin hydrogels decreased with an increase in the glutaraldehyde concentration. It is obvious that the higher glutaraldehyde concentration, the higher the crosslinking extent of hydrogels prepared. Because there is no good method available for directly determining the extent of crosslinking, we regarded the water content of hydrogels as an experimental parameter reflecting their crosslinking extent. In the present study, four types of hydrogels with different water contents were used. Glutaraldehyde is a crosslinking agent which is allowed to chemically crosslink between amino groups of gelatin molecules. It is possible that the crosslinked gelatin hydrogel is degraded through enzymatic cleavage of gelatin molecules rather than through their simple hydrolysis. The higher the extent of hydrogel crosslinking, the less enzyme accessibility to gelatin for cleavage, leading to a reduced hydrogel degradation. Indeed, in the enzyme present in vivo, it was found that chemically crosslinked gelatin films of lower water content were degraded more slowly than those of higher water content [14]. The degradation period of hydrogels could be regulated by changing the crosslinking extent and four types of hydrogels were degraded in the skull defect over the time range from 2 weeks to 4 months, irrespective of the bFGF impregnation. The shape and size of hydrogel discs did not change by the bFGF impregnation process, irrespective of their bFGF loading and water content.

3.2. Histological evaluation

Figure 3 shows histological sections of the skull defects 12 weeks after treatment under different conditions. Insignificant bone regeneration and remarkable ingrowth of soft connective tissues at the defect were observed for rabbits when treated with PBS, empty gelatin hydrogels and free bFGF (Fig. 3A–C). bFGF-free gelatin hydrogels neither induced bone formation nor interfered with bone regeneration at the skull defect. On the contrary, the skull defect of rabbits treated with gelatin hydrogels incorporating 100 μg of bFGF was filled with a newly regenerated bone tissue and almost closed at 12 weeks after implantation. Comparison of Fig. 3E with Fig. 3F revealed that osteoblasts were seen in an active form around the edge of bone treated with bFGF-incorporating hydrogels in contrast to free bFGF treatment. In Fig. 3F, tissue around the bone edge of defect treated with bFGF-incorporating hydrogel composed of woven bone, whereas the free bFGF-treated tissue composed of lamellar bone (Fig. 3E). This histological examination indicates that in the former tissue, bone regeneration was in progress even after 12 weeks of treatment. However, the regeneration process had already been over in the latter tissue and the tissue returned to originally static appearance. No cartilaginous tissues were histologically observed during the process of bone formation by bFGF-incorporating gelatin hydrogels. Bone regeneration induced by the hydrogel-incorporating bFGF was not initiated from the central portion of the defect but from the edge.

3.3. Mineral deposition at skull defects

Table 1 summarizes results of the BMD measurement for the skull defects of rabbits 8 and 12 weeks after different treatments. The BMD of intact rabbit skulls was $120 \pm 10\ \mathrm{mg\ cm^{-2}}$. As is seen, the results are in good agreement with the photographs in Fig. 3. It is clear that the bFGF-incorporating gelatin hydrogel enhanced significantly the BMD of skull defect, both for the hydrogels of water contents of 85 and 98%. BMD was significantly higher for rabbits treated with the 85% hydrogel than the 98% one. On the other hand, free bFGF did not exhibit any significant bone regeneration, although the BMD tended to be somewhat high compared with that of PBS-treated, control rabbits. The BMD at the defect of rabbits treated with bFGF-free, empty gelatin hydrogels was similar to that of control rabbits, indicating that implantation of the hydrogels in the defect did not disturb bone regeneration at the site. A similar trend was

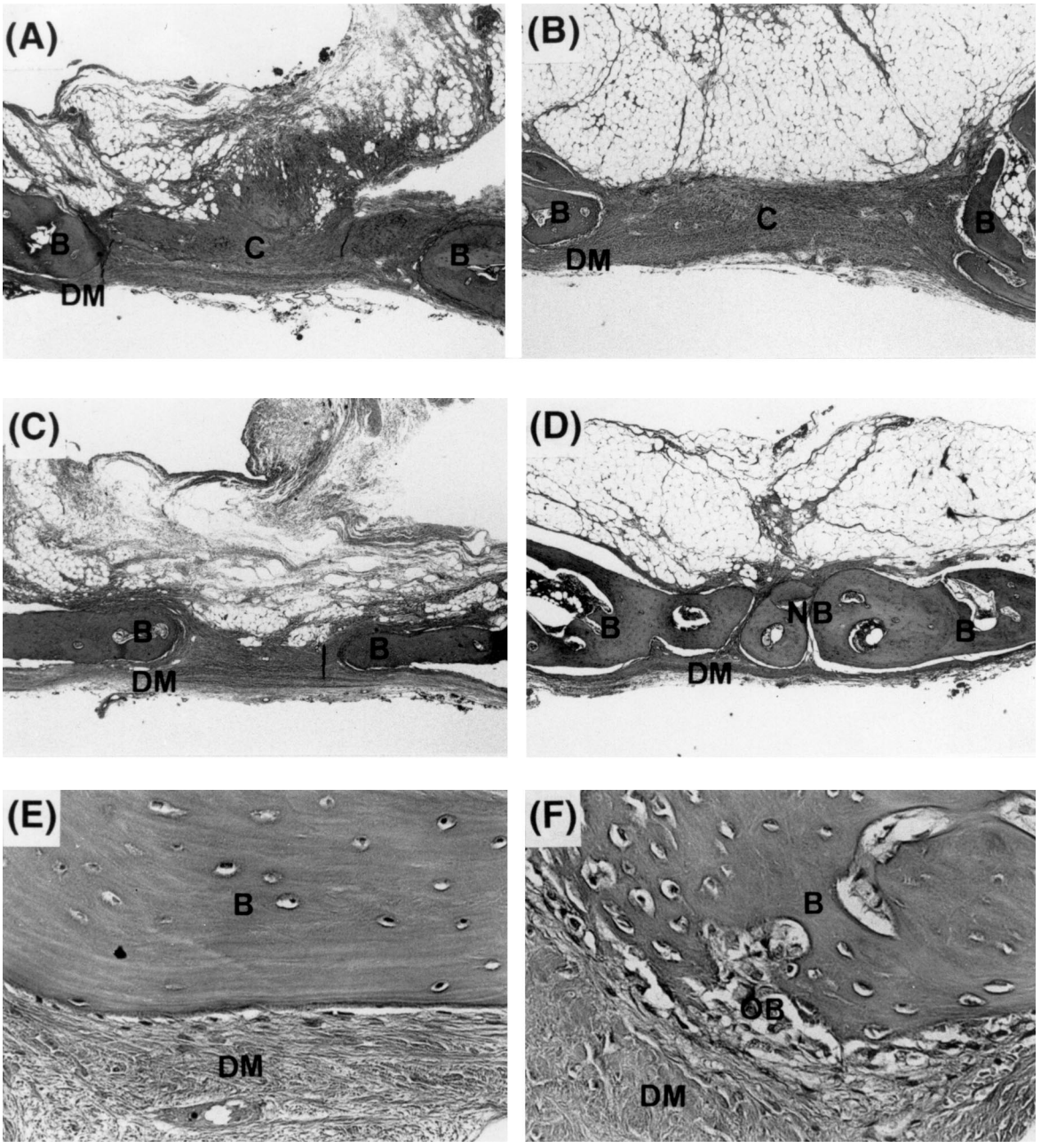

Fig. 3. Histological cross-sections around the skull defect of rabbits 12 weeks after treatment with PBS (A), an empty gelatin hydrogel with a water content of 85% (B), 100 μg of free bFGF (C, E) and a bFGF-incorporating gelatin hydrogel with a water content of 85% (D, F): B; bone, DM; dura mater, C; connective tissue, NB; new bone and OB; osteoblast. (A, B, C and D; HE staining, ×40, E and F; HE staining, ×400)

observed for bone formation 8 weeks after implantation, although the efficacy of the bFGF-incorporating hydrogels in enhancing bone regeneration was not as clear as that of hydrogels implanted for 12 weeks. The BMD values ranged from 50.8 to 55.6, 50.2 to 58.3, 60.3 to 67.3, and 76.4 to 92.5 mg cm^{-2} at 4 days and 1, 2 and 4 weeks after treatment, respectively, no significant difference in the BMD being observed between experimental groups.

Figure 4 shows the effect of bFGF dose on the bone formation induced by free bFGF and bFGF-incorporating gelatin hydrogels. Apparently, incorporation of bFGF in gelatin hydrogels was effective in enhancing bone formation at the skull defect. bFGF-incorporating gelatin hydrogels significantly enhanced the BMD of skull defect over the bFGF dose range studied. On the other hand, free bFGF did not exhibit any significant bone regeneration except for the highest dose that produced a somewhat high BMD compared with that of rabbits treated with PBS and empty gelatin hydrogels.

Table 1
Bone mineral densities (BMD) at a skull defect of rabbits 8 and 12 weeks after various treatments

Treated with	Water content (%)	BMD (mg cm^{-2})	
		8 weeks	12 weeks
bFGF-incorporating gelatin hydrogel (100 μg bFGF/rabbit)	85	102.0 ± 6.13**	115.9 ± 6.97***†‡
	98	100.8 ± 11.3*	101.5 ± 10.6**†
Free bFGF (100 μg bFGF/rabbit)	—	94.0 ± 11.4	82.6 ± 14.2
bFGF-free, empty gelatin hydrogels	85	82.7 ± 9.11	76.6 ± 9.77
	98	85.8 ± 16.1	80.5 ± 7.75
PBS	—	82.2 ± 8.47	74.1 ± 9.62

The BMD of intact rabbit skulls was 120 ± 10 mg/cm^2.
***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$, against PBS-treated rabbit groups.
††: $P < 0.001$, †: $P < 0.05$, against free bFGF-treated rabbit groups.
‡: $P < 0.01$ against bFGF-incorporating hydrogel (98%) rabbit groups.

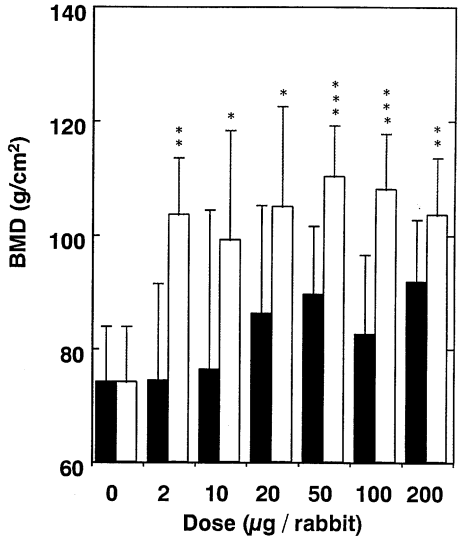

Fig. 4. The effect of bFGF dose on the bone mineral density (BMD) around the skull defect of rabbits 12 weeks after treatment with PBS (■) and gelatin hydrogels with or without bFGF (□). The water content of hydrogels was 85%. Each experimental group consisted of 6 defects from 6 different rabbits. ***, **, *: $P < 0.001$, $P < 0.01$, $P < 0.05$ vs. PBS-treated groups.

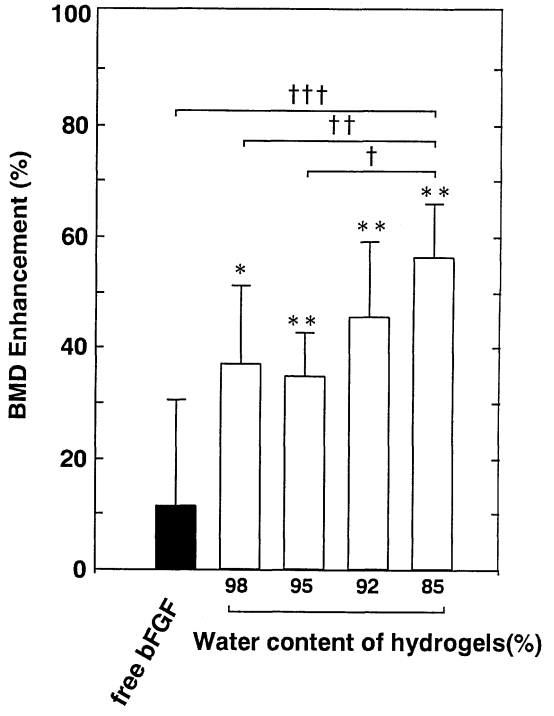

Fig. 5. Enhancement of the bone mineral density (BMD) around the skull defect of rabbits 12 weeks after treatment with free bFGF (■) and bFGF-incorporating gelatin hydrogels of various water contents (□) (100 μg bFGF/defect). Each experimental group consisted of 6 defects from 6 different rabbits. **, *: $P < 0.001$, $P < 0.01$ vs. PBS-treated groups. †: $P < 0.05$ vs. groups treated with bFGF-incorporating hydrogels with a water content of 95%. ††: $P < 0.01$ vs. groups treated with bFGF-incorporating hydrogels with a water content of 98%. †††: $P < 0.001$ vs. groups treated with free bFGF.

Figure 5 shows the influence of hydrogel water content on the bone regeneration induced by bFGF-incorporating gelatin hydrogels. It is again seen that incorporation of bFGF in the gelatin hydrogels enhanced the BMD at the skull bone defect to a significantly high extent compared with free bFGF, irrespective of their water content. However, the BMD enhancement depended on the water content of bFGF-incorporating gelatin hydrogels; the hydrogels of lower water content showed higher BMD than those of higher water content.

3.4. Osteoblast recruitment at the bone defect

Figure 6 shows the time course of the number of osteoblasts locating near the bone edge of the skull defect of

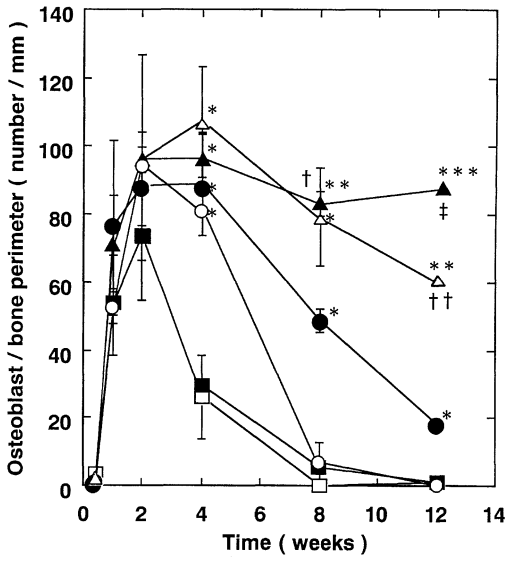

Fig. 6. Time course of the osteoblast number around the skull defect of rabbits. The skull defect was treated with free bFGF (○), bFGF-incorporating gelatin hydrogels with water contents of 98 (●), 92 (△) and 85% (▲) and PBS (□), empty gelatin hydrogels with a water content of 92% (■) (100 μg bFGF/defect). Each experimental group consisted of 6 defects from 6 different rabbits. **, *: $P < 0.001$, $P < 0.01$ vs. PBS-treated groups. ††, †: $P < 0.01$, $P < 0.05$ vs. groups treated with bFGF-incorporating hydrogels with a water content of 98%. ‡: $P < 0.01$ vs. groups treated with bFGF-incorporating hydrogels with a water content of 92%.

rabbits after different treatments. Interestingly, during the initial 2 weeks, the time course of cell number was similar among differently treated rabbits. In histological examination, no difference between the rabbit groups treated with free bFGF and bFGF-incorporating gelatin hydrogels was observed in the distribution profile and the number of the cells which was positively stained with alkaline phosphatase during the initial 1 and 2 weeks. The profile of the cell number thereafter depended on the treatment type. The osteoblast number increased up to 2 weeks and then decreased for both the rabbit groups treated with PBS and empty gelatin hydrogels. No difference in the time profile of osteoblast number was observed between empty gelatin hydrogels with different water contents (data not shown). Free bFGF treatment prolonged the retention period of cells by a few weeks, but the number returned to the basal level on week 8. On the contrary, the rabbit groups treated with bFGF-incorporating gelatin hydrogels did not exhibit the decrease in the cell number as rapid as that of free bFGF treatment, retaining a significantly high level of cell number over the time range studied, irrespective of the hydrogel type. However, the retention period of the enhanced cell level tended to prolong with a decrease in the hydrogel water content.

4. Discussion

The ability of skull bones to regenerate by themselves is recognized to be much lower than that of long bones [12]. If this is true, closure of a flat bone defect with a newly formed bone would be practically impossible, unless protein factors capable of strongly differentiating immature cells into osteoblastic cells, such as bone morphogenetic protein (BMP) [15] and transforming growth factor-β1 (TGF-β1) [16], are provided. In fact, the combination of BMP with a matrix was found to be effective in filling experimental bone defects of animals [17–19], although few studies have been reported on primates with promising results [20]. bFGF is a growth factor to trigger proliferation of capillary endothelial cells in addition to osteoblasts [21], unlike BMP. Weiss et al. reported that bFGF stimulated the cells involved in osteogenesis and angiogenesis in a vascularized bone graft [22]. Indeed, angiogenesis was observed around the skull defect of bFGF-treated rabbits during the initial period. Although bFGF does not have such a strong activity for cell differentiation as BMP, the present study clearly demonstrates that combination with a good carrier enables bFGF to enhance its osteogenic function, leading to closure of a skull defect. Animal experiments with ^{125}I-labeled bFGF or gelatin demonstrated that the retention period of bFGF in the body was in good accordance with that of gelatin, indicating that bFGF is released as a result of in vivo degradation of the hydrogel matrix [23]. This sustained release of bFGF from the gelatin hydrogel will result in enhanced bone regeneration of skull defects.

Histological and DEXA examinations demonstrated that bFGF-free gelatin hydrogels did not exhibit any inherent properties to promote and interfere with bone regeneration (Fig. 3 and Table 1). The highly ordered structure of collagen is known to play an important role in the calcification process [24]. Thus, it is unlikely that a denatured collagen, gelatin positively functions in bone formation. All types of implanted gelatin hydrogels were degraded with time finally to completely disappear from the defective site 12 weeks after implantation, although the degradation rate depended on their water content. It is possible that soft tissues grew into the defect to fill the dead space formed with hydrogel degradation. If the hydrogels are degraded at a rate lower than that of bone growth, the bone regeneration at the skull defect will be physically interfered with the hydrogel. Such an interference was not observed for the most slowly degrading hydrogels with the water content of 85%. The threshold period of hydrogel degradation to start interfering bone regeneration is unclear at present.

bFGF-incorporating gelatin hydrogels of lower water content showed higher BMD than those of higher water content (Fig. 5). Slower degradation of the hydrogels is undoubtedly due to a larger extent of crosslinking. Since

bFGF is released from the gelatin hydrogels as a result of gelatin biodegradation, slower degradation of the hydrogels will prolong the release period of bFGF, resulting in more enhanced BMD.

It should be noted that bFGF-incorporating gelatin hydrogels of lower water content maintained the increased cell level for a longer period than those of higher water content. This can be explained in terms of bFGF release. The hydrogels of lower water content will be degraded more slowly, leading to in vivo bFGF release for a longer time period. This prolonged bFGF release possibly gives a continuous stimulus to osteoblast proliferation which is responsible for the prolonged retention of the osteoblast number at a high level (Fig. 6). We, therefore, believe that the different cell numbers lead to the different BMD among the bFGF-incorporating gelatin hydrogels with various water contents (Fig. 5). Such a feature of bFGF-incorporating hydrogels must have enhanced and retained the bFGF activity for bone regeneration, effectively filling the bone defect of rabbit skull with the newly formed bone.

Bone regeneration induced by the hydrogel incorporating bFGF was not initiated from the central portion of the defect but from the edge. This is probably because the precursor cells of osteoblasts are necessary to initiate bone formation. It is well recognized that the precursor cells mainly exist in the periosteum and are differentiated into osteoblasts to induce bone formation [25]. In this case, osteoblastic cells positively stainable with alkaline phosphatase, were not observed in the central portion of the skull defect but along the defect margin, resulting in bone regeneration from the bone edge, which is similar to that observed for carrier matrices combined with other osteoinductive growth factors [17, 26, 27].

5. Conclusions

Gelatin hydrogels incorporating bFGF enhanced bone regeneration in rabbit skull defects, finally closing the defects whereas free bFGF was ineffective. The enhanced bone regeneration induced by the hydrogels depended on their degradation rate. More slowly degrading hydrogels achieved in vivo long-term bFGF release and retained osteoblasts number high in the bone defects for a long period, leading to enhanced bone regeneration compared with faster degrading hydrogels.

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