Combination of the RANKL-binding peptide W9 and bFGF induces bone regeneration in the rat calvarial defect model

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Abstract

The therapeutic use of a single molecule(s) for efficacious induction of bone regeneration may be ineffective owing to the various conditions prevailing in bone defect sites. Therefore, a combination therapy for stable bone regeneration has been developed. One of the RANKL-binding peptide was W9 (YCWSQYLCY). It was known to enhance osteoblast differentiation. Contrastingly, basic fibroblast growth factor (bFGF) promotes bone formation by enhancing osteoblast proliferation. This study was aimed at investigating the ability of co-administration of W9 and bFGF. Proliferation and differentiation assays were performed in vitro. A mesenchymal cell line was used in evaluating effects of the bFGF and W9 combination (bFGF/W9) on proliferation. One reason for using a mesenchymal stem cell line is that in primary osteoblasts W9 does not increase proliferation and only differentiates. Primary osteoblasts were used in evaluating the effect of bFGF/W9 on differentiation. W9 enhanced the bFGF-induced increase in proliferation and early osteoblast differentiation marker level. However, bFGF/W9 was not effective as a late osteoblast differentiation marker. For *in vivo* analyses, Wistar rats were used, and gelatin hydrogel containing bFGF with or without W9, was applied to the calvarial defect. The rats were sacrificed, and newly formed bones were analyzed radiographically and histologically. The bFGF/W9 group showed bone formation compared to other groups. Altogether, it was suggested that bFGF/W9 stimulates bone regeneration in post-operative bone defects of the maxillofacial region.

Introduction

For appropriate bone regeneration, the use of single molecule(s) in therapy may be ineffective because of the variety of conditions present at the bone defect sites. When the size of the bone defect exceeds larger than a critical size, a long period of time is required to regenerate bone if single molecule(s) is used. Additionally, there are concerns regarding the safety and practicality of growth factors, and side effects such as abnormal immune reactions occur [1,2]. Furthermore, various agents act not only osteogenesis but also angiogenesis, vasculogenesis and inflammation modulation [1,3-6]. So, it is considered desirable that two kinds of agents are used for bone regeneration [7]. Therefore, the effects of the co-administrations of bone forming agents such as bFGF, prostaglandin E2 agonist, BMP-2, TGF-β1, and bone-forming peptides has been investigated toward clinical applications [8-13]. Among those bone forming agents, a peptide drug with a molecular weight around 1,000 ~ 2,000 is expected as a suitable candidate for development of bone-anabolic drugs since it combines the merits of both low-molecular-weight drugs and antibody-drugs; high specificity to drug target, low adverse effects, feasibility for inhibiting protein-protein interactions, availability of synthesizing agent, and low production cost [14]. Especially the in vivo effects of bFGF on bone formation

are apparent, its clinical usage have been studied intensively in animal experiment. Recently, W9 is found to promote bone formation by promoting osteoblast differentiation [15-17]. It was originally designed to mimic the critical binding site of tumor necrosis factor (TNF)-α on TNF type1 receptor, and binds the receptor activator of NF-κB ligand (RANKL), thereby inhibiting osteoclastogenesis [18]. On the other hand, bFGF is known to stimulate bone formation by promoting osteoblast proliferation [19,20]. Therefore, we hypothesize that co-administrations of bFGF and W9, the proliferation- and the differentiation-stimulators of osteoblasts, could be an appropriate combination for bone regeneration. In this study, we investigated the

effects of co-administration of W9 and bFGF on cell proliferation, osteoblast differentiation and bone regeneration using rat calvarial defect model.

Materials and methods

Reagents

W9 peptide (YCWSQYLCY) and the control peptide (CtrP; YCWSQNLCY) were purchased from the American Peptide Company (Sunnyvale, CA, USA). These peptides have similar size and structure, but CtrP binds to RANKL with a lower affinity than W9 [21]. In addition, bFGF was provided by Kaken Pharmaceutical Co. Ltd. (KAKEN, Tokyo, Japan). Gelatin hydrogel (GH) sheets (type pI5) and GH granules (type pI9) were used as bFGF and peptide carriers, respectively [22,23]. However, in the GH-W9 group, W9 was dropped onto GH sheets (type pI9) instead of GH granules to prevent outflowing from the defect site.

Cell culture

Proliferation and differentiation assays were conducted *in vitro*. In the proliferation assay, the mesenchymal cell line, C3H10T1/2 (a murine pluripotent mesenchymal cell line [RIKEN, Ibaraki, Japan]) was used to evaluate effects of combination therapy with bFGF/W9. These cells were seeded at a density of 5×10^3 cells/well in a 96-well plate, and cultured in basal medium eagle (BME; Life Technologies/ Gibco, New York, USA) containing 10% fetal bovine serum (FBS; Hana-Nesco Bio, Brisbane, Australia), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich) in the presence or absence of either 1 ng/ml bFGF, 200 μM W9, or 200 μM CtrP for 48 h. Cell Count Reagent SF (Nacalai Tesque, Inc., Kyoto, Japan) was added to each well, cells were incubated for 2 h, and the optical density of each well was measured at 450 nm using a microplate reader (iMarkTM Microplate Reader; Bio-Rad, Hercules, CA, USA).

The differentiation assay was performed as described previously [24]. Briefly, primary osteoblasts isolated from calvariae of one-day-old mice were seeded at a density of 5×10^4 cells/well in a 24-well plate (3.6 cm^2/ each well, Sumitomo Bakelite Co., ltd.) with either 10 ng/ml bFGF, 200 μM W9, or 200 μM CtrP. The alkaline phosphatase (ALP) staining was performed on day 6, and the von Kossa staining was performed on day 21 of osteoblast culture, and the ALP- and von Kossa-positive areas were measured using an ImageJ analysis system [25].

Animals

Twenty 9-week-old male Wistar rats were used (weight: 270–310 g; Sankyo Labo Service, Tokyo, Japan). All experimental protocols were reviewed and approved by the animal research committee of the Tokyo Medical and Dental University (Tokyo, Japan; authorization number 0170324A). The rats were housed in individual stainless steel cages under controlled temperature conditions, with free access to standard rat feed and water.

Rat calvarial defect model

Before the procedures, rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg). The skin around the surgical area was shaved, and cleaned with 70% ethanol. Bleeding was suppressed by using local infiltration anesthesia (2% xylocaine, 1:80, 000 adrenaline).

Twenty rats were randomly divided into five groups (n = 4) based on materials incorporated on GH discs: 1) carrier alone; 2) bFGF (25 μg); 3) W9 (0.56 mg); 4) bFGF (25 μg) + W9 (0.56 mg); 5) bFGF (25

μg) + CtrP (0.56 mg). The GH disc containing each reagent(s) was placed on a calvarial critical-size defect (5 mm in diameter) on the right parietal bone, generated using a biopsy punch (Kai Industries, Gifu, Japan). However, bFGF and W9 have a different carrier type and were administered using 2 types of GH: the GH sheet with bFGF was placed in a previously generated defect, onto which a GH granule with W9 or CtrP was dropped. All rats were subcutaneously injected with calcein (10 mg/kg) and alizarin (30 mg/kg), on day 15 and 20, respectively. Twenty-eight days after surgery, all rats were sacrificed after overdose of pentobarbital (100 mg/kg). Subsequently, the cranial bone of rats was removed and fixed in PBS-buffered formaldehyde (3.7%) solution (pH 7.4) for 2 days at 4°C, washed with PBS for 1 day, and used for radiographic analysis.

Radiographic assessment of the calvarial defect

Three-dimensional reconstruction images of newly formed bones at the defective areas were obtained by micro-computed tomography (μCT) (Scan Xmate-E090; Comscan Tecno, Yokohama, Japan). Area of newly formed bone at the bone defects was calculated from μCT-reconstructed images by using an ImageJ analysis system [25].

Histological assessments and bone histomorphometry of the calvarial defect

For histological analysis, undecalcified frozen sections (5-μm thick) were prepared using a microtome (CM3050sIV; Leica Biosystems, Nussloch, Germany), as described previously [26]. Images of fluorescently labeled sections were used to confirm the form of bone formation. Bone histomorphometry was performed in a rectangular region of interest (3.88×5.50 mm^2) of the whole defective site. Mineralizing surface (MS) and mineral apposition rate (MAR) were measured using a KS400 image analysis system, as previously described [24,27]. Local bone formation activity was defined as multiplication of MS and MAR.
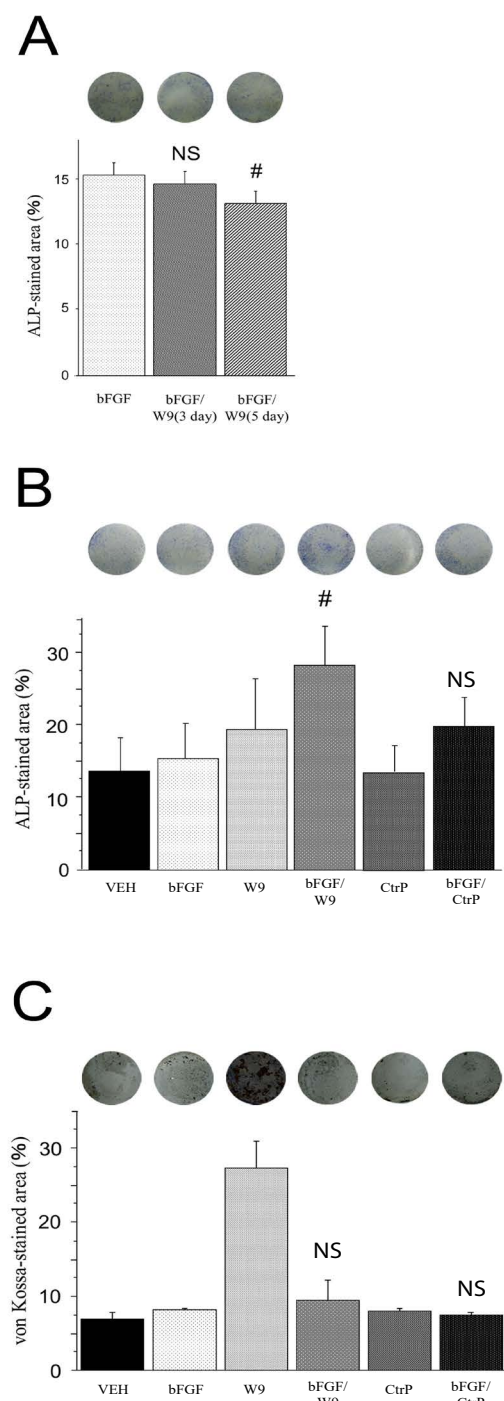
Statistical analysis

Data are presented as mean ± standard deviation (SD). For both in vivo and in vitro studies, the statistical significance of differences among groups was assessed using one-way ANOVA. Fisher's PLSD post hoc test was performed upon detection of significant F-values. $P < 0.05$ was considered to be statistically significant.
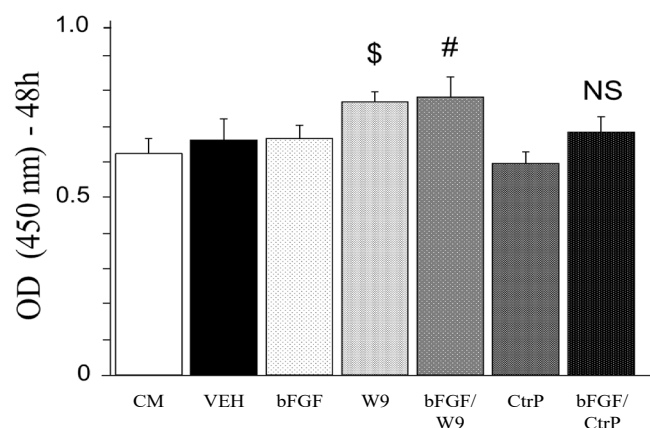
Results

The bFGF/W9 combination promoted early osteoblast differentiation

Osteoblast culture was initiated with only bFGF, and W9 was administered after 3 and 5 days. ALP staining was performed on day 6 (Figure 1A). The ALP-positive area of the bFGF/ (after using W9) group was not significantly larger than that of the bFGF group. The ALP- and von Kossa-stained areas were measured respectively after 6 and 21 days to evaluate differentiation of primary osteoblasts (Figures 1B & 1C). W9 increased the bFGF-induced ALP-positive area upon simultaneous addition of W9 and bFGF to the same well (Figure 1B). However, the bFGF/W9 combination group was not effective as per the von Kossa-positive area (Figure 1C). These additive effects were suppressed by CtrP, which binds RANKL with a lower affinity than W9.

In vitro assays suggested that W9 may promote bFGF-induced osteogenesis by promoting mesenchymal cell proliferation and early osteoblast differentiation.

Figure 2. RANKL-binding peptide W9 enhanced bFGF-induced mesenchymal cell proliferation for 48h. C3H10T1/2 cells were seeded with 5×10^3 cells/well. Cell proliferation was assayed using Cell Count Reagents SF. W9 and Control peptide were used at a concentration of 200 μM, and bFGF was used at a concentration of 1 ng/ ml. The data are expressed as the means ± SD (n=5 for each group). CM: condition medium group. $ P < 0.05 vs. CM; # P < 0.05 vs. bFGF; NS: no significant difference.

W9 stimulated bFGF-induced cell proliferation

Mesenchymal cell line proliferation was evaluated *in vitro*. In our experiment, we used condition medium (CM), which constituted BME with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in all groups. We performed two types of experiments—one, wherein bFGF and W9 were added simultaneously, and the other, wherein W9 was added one day later.

The bFGF/W9 combination group showed enhanced C3H10T1/2 cell proliferation on day 2 in the former experiment (Figure 2), but not in the latter. Furthermore, CtrP did not enhance bFGF-induced proliferation.

These results suggested that these two agents are effective when used simultaneously. In this experiment, it was impossible to obtain a significant difference between the CM group and the bFGF group, but it was able to show the tendency of proliferation.

W9 stimulated bone formation at the rat calvarial defect site

Rats were sacrificed on day 28 post-operation, and local bone formation on a calvarial critical-size defect was first analyzed radiologically. Micro CT images revealed that the bFGF group showed local bone formation near the defect margins (Figure 3A). W9 group and carrier alone group did not show increased bone formation in defective areas. The bFGF/W9 combination greatly enhanced marginal and bone formation in defective areas. However, CtrP seemed to have a less stimulatory effect on bFGF-induced bone formation. Newly formed bone area (%) at the defect site was calculated by using an ImageJ analysis system as previous described [25]. Newly formed bone area was significantly higher bFGF/W9 group than in the other experimental groups (Figure 3B).

The local bone formation was histologically analyzed. To visualize sequential changes in local bone formation at the calvarial defect site, fluorescent dyes—calcein and alizarin—were subcutaneously injected on day 15 and 20 post-operation, respectively. The results of HE staining were similar to the result of fluorescent labeling, whereby HE-stained area for bFGF/W9 group was only detected ectopically-like bone formation in all groups (Figures 4A & 4B). In addition, local bone formation activity, which indicates the total calcified area that is
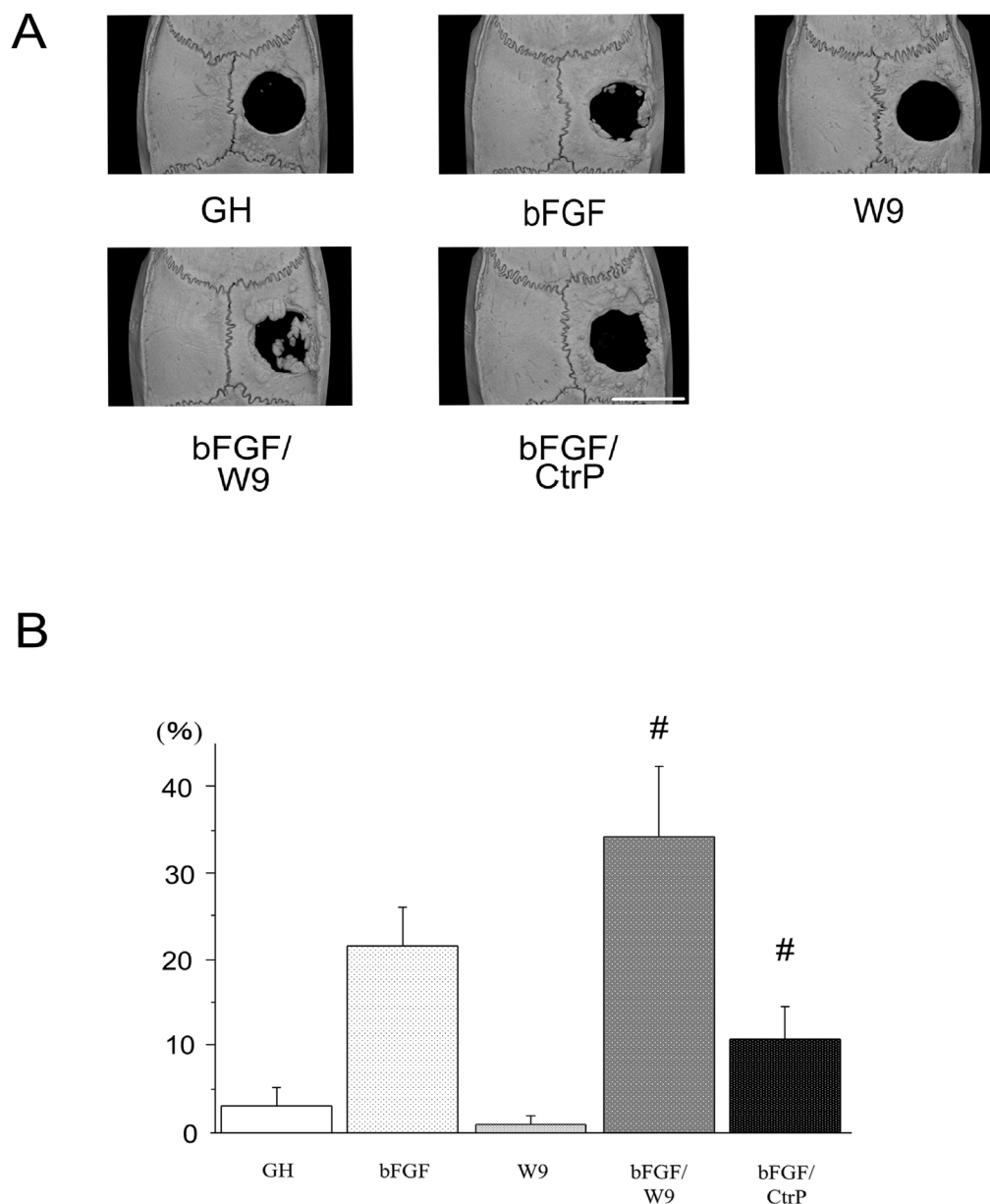

Figure 1. RANKL-binding peptide W9 enhanced early osteoblast differentiation. Primary osteoblasts were seeded with 5×10^4 cells/well. W9 and control peptide were used at a concentration of 200 μM, and bFGF was used at a concentration of 10 ng/ ml. (A) Representative image of experiments in which W9 was added into bFGF on days 3 and 5. The percentage of ALP-stained area was calculated in each well. The data are expressed as the means ± SD (n=3 for each group). (B) ALP-positive primary osteoblasts in culture on day 6. The osteoblasts were fixed and ALP staining was performed. Images of the ALP-staining cells were shown. The percentage of ALP-stained area was calculated in each well. (C) Cells were cultured for 21 days and von Kossa staining was performed. Images of the von Kossa-staining cells were shown. The percentage of von Kossa-stained area was calculated in each well. The data are expressed as the means ± SD (n=6 for each group). bFGF: bFGF group, bFGF/W9: bFGF and W9 group, CtrP: control peptide group, bFGF/ CtrP: bFGF and the control peptide group, VEH: vehicle control group. NS: no significant difference. # P < 0.05 vs. bFGF; NS vs. bFGF.

Figure 3. RANKL-binding peptide W9 promotes bFGF-induced bone formation in a rat calvarial defect model. (A) Micro-computed tomography (μCT) reconstruction images of the whole mount of calvariae. Scale bar represents 5 mm. (B) The percentage of newly formed bone area in defect was shown. The data are expressed as the means ± SD (n=4 for each group). # P < 0.05 vs. bFGF.

produced in the region of interest in a single day, also showed similar results to those of the mineralizing surface (Figure 4C).

Discussion

W9-induced augmentation of cell proliferation may be involved in stimulation of early osteoblast differentiation by simultaneous addition of bFGF and W9

We have investigated the combined effects of bFGF and the RANKL-binding peptide W9, which are known to stimulate osteoblast proliferation and differentiation, respectively [15-17,28,29]. We first hypothesized that the bFGF works as an inhibitor of osteoblast differentiation induced by W9 when two agents were added to the culture media at the same time since proliferation stimulator usually inhibits differentiation [30]. Although this was the reason why we decided to add W9 to the osteoblast culture several days after the bFGF stimulation, our differentiation assay revealed that the simultaneous addition of bFGF and W9 got the better augmentation of early osteoblast differentiation compared to the condition that W9 was added later (Figure 1). This result is consistent with the results that bFGF increases proliferation and early differentiation activity and suppresses late differentiation [31]. Then, we thought W9 might affect to cell proliferation as well as differentiation. Actually, we found at the first time that W9 stimulated cell proliferation in the mesenchymal cell line (Figure 2). W9 has been already shown to stimulate mTORC1 signaling in ST-2 osteoblastic cell line [17], and mTORC1 signaling is important in mesenchymal cell proliferation [32]. W9 might promote cell proliferation through mTORC1 signaling. Since the control peptide
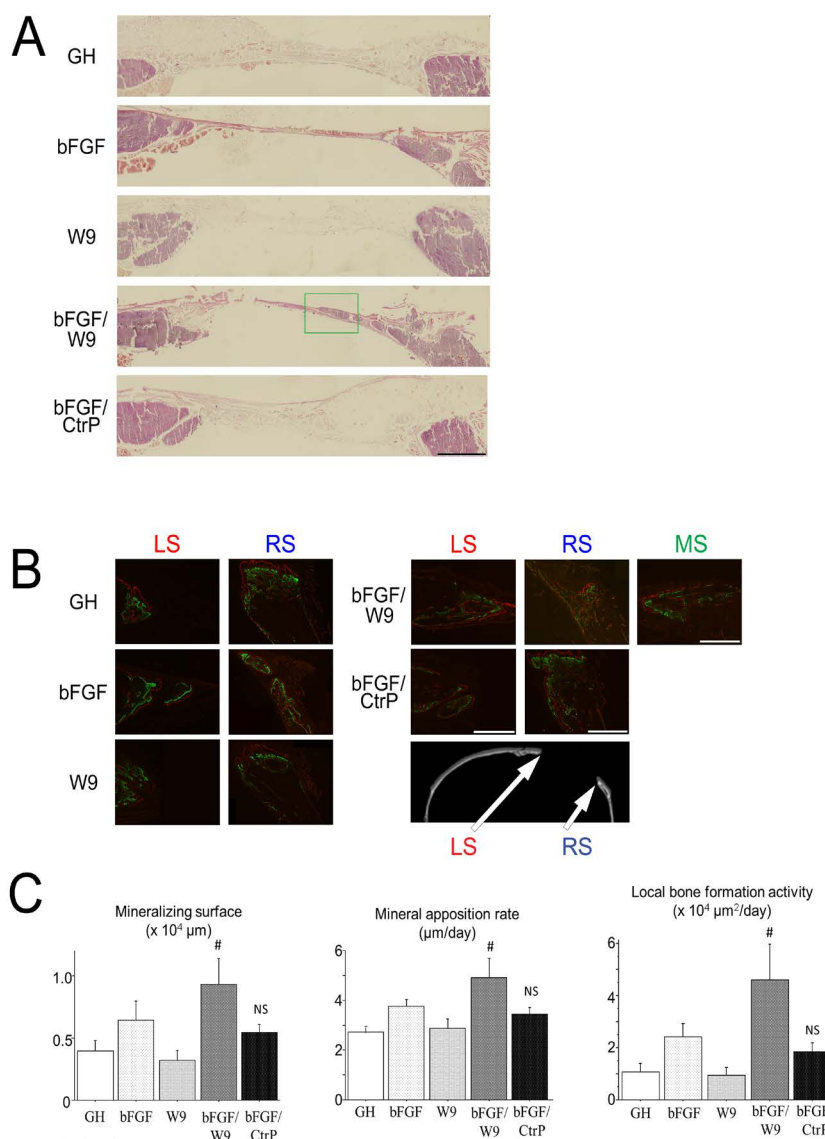
Figure 4. RANKL-binding peptide W9 could promote bFGF-induced bone formation in vivo.All sections were prepared to pass through the center. (A) Representative HE staining images of decalcified frozen sections of defect in the right side of calvaria. Scale bar represents 1 mm. (B) Representations of the fluorescently stained decalcified frozen sections of the calvarial defect. The panels indicate higher magnification views of the LS, RS and green frame (denoted as MS; middle side). Marginal bones are depicted as the red side (LS) and the blue side (RS). Center bone is only depicted in MS of the FGF/W9 group. Calcein labeling (administered on day 15) is shown in green, and alizarin labeling (administered on day 20) is shown in red. Representations of the staining are shown. Scale bar represents 500 μm. (C) Quantitative analyses of bone formation activity were performed using standard bone histomorphometric measurement techniques based on the calcein- and alizarin red-labeled surface in the ROI (as described in Materials and methods section), centering on the area of regenerated bone. Local bone formation activity was calculated as (mineralizing surface) × (mineral apposition rate). The data are expressed as the means ± standard deviation for each group (n=4). # P < 0.05 vs. bFGF.

of W9, which has a lower affinity to RANKL compared to W9, did not stimulate mesenchymal cell proliferation, suggesting that the affinity to RANKL might be involved in the stimulatory mechanism of W9 on cell proliferation. Further studies are necessary to clarify the stimulatory mechanism of W9 on cell proliferation.

W9 promotes bFGF-induced bone formation

In this study, we have shown that the administration of W9 with bFGF promotes bone formation in critical-sized (5 mm in diameter) rat calvarial defect. The reason for using this experimental model is that rat calvaria is similar to human mandible as both develop through intramembranous bone formation and both demonstrate limited innate regenerative potential [33,34]. It has been reported that 0.3%

(30 μg) bFGF increases bone formation in the same model, as that used in this study, using absorbable collagen sponge carrier, but bone formation was not observed in the whole defect [35].

Therefore, we used the GH carrier in in vivo studies. The GH carrier holds the agent through molecular interactions, such as hydrogen binding, hydrophobic binding, and Coulomb's force, and the agent is released slowly as the GH is degraded [36]. This carrier is known to achieve controlled release of bFGF [37] and peptide drugs [16,29]. Therefore, the GH has good affinity with drug regardless of hydrophilicity or hydrophobicity. Since the isoelectric points of bFGF and W9 are different, we have used two different types of GH carriers, pI5 and pI9, respectively [16,29,38]. 25 μg bFGF, which was used in in

vivo experiments, was decided to be appropriate for bone formation in this model. In a previous study, the skull defect in rabbits was filled with newly regenerated bone and was almost closed 12 weeks after implantation, after treatment with 100 µg bFGF-incorporated GH [36]. Additionally, it is reported that bone regeneration induced by bFGF-incorporated GH was not initiated from the central portion of the defect but from the edge [37]. First, to combine bFGF/W9, we assessed the amount of bone formation occurring with minimal quantity of bFGF. After determining the dose of bFGF, the dose of W9 was determined with reference to previous reports [16,17]. Combination of 25 µg bFGF and 0.56 mg W9 (bFGF/W9 group) showed significant enhancement of bone formation compared with that of other groups. Newly formed bone was scarcely recognized at twice the dose in in vivo experiments, and administration of the same amount resulted in bone formation more than that by bFGF alone. Next, to confirm the difference in RANKL binding capacity, we used CtrP, which is the peptide containing W9 mutation, and results revealed that they bind with a lower affinity to RANKL than to W9. The bFGF/CtrP group resulted in significant suppression of bone formation compared to that in the bFGF/W9 group. However, bone formation was scarcely observed in GH carrier and GH + W9 groups. From these results, W9 might promote bFGF-induced osteogenesis in a RANKL-affinity dependent manner. Until now, bone formation was not expected to occur due to bFGF in experiments using the rat calvarial defect model. However, promotion of osteogenic effect by bFGF was confirmed by the concomitant use of W9. The results of this experiment revealed bone formation in the edges and the ectopic area. A previous study reported that bFGF alone does not induce ectopic bone formation (16, 80 and 400 ng; and 2, 10, and 50 µg bFGF), but induces the formation by addition of 2 µg bFGF in rat calf muscle [39]. Briefly, bone formation requires BMP-2. In the dog periodontal model, bFGF promoted the expression of BMP-2 to induce osteoblastic differentiation and bone formation [40]. Considering these results, our data suggested that BMP-2 was expressed when bFGF was used, and BMP-2-like action was enhanced by using W9 resulting in bone formation. This is consistent with the report that the RANKL-binding peptide enhances the action of BMP-2, compared with past mouse calvarial defect experiments. Moreover, as mentioned above, it is thought that it can support against the late differentiation effect of bFGF can't be expected. Further, it is necessary to confirm the mechanism of bone formation and BMP-2 expression when bFGF and W9 are combined.

Perspectives of combination therapy with two different agents

We used two types of agents bFGF and W9 with a controlled release. The dual controlled release system is one of the attractive methods employed for bone regeneration [7]. In this study, osteoblast proliferation was carried out by bFGF, and W9 might have enhanced bFGF-induced osteoblast differentiation. However, more complicated reactions must be occurring during the process. If two types of agents are used, synergistic effect is just a possibility, and if they are used at high concentrations, there is a possibility of inhibition of bone regeneration. It is difficult to decide a ratio, but good bone regeneration can be expected when the ratio is appropriate.

The synergistic effects of two different agents on proliferation might be one of the reason why the combination of W9 and bFGF could stimulate bone regeneration compared to the administrations of W9 or FGF alone. Since bFGF stimulate proliferation through MAPK pathway and W9 might be related to the mTORC1 activation on proliferation as described earlier, the different stimulatory mechanisms could generate additively effects for local bone formation. As suggested in this study

(Figure 1), the RANKL-binding peptide could stimulate osteoblast differentiation under the RANKL-affinity dependent mechanism [15,41]. The combination therapy of W9 and bFGF also exerts its effects under the RANKL-affinity dependent mechanism (Figure 4), suggesting that two different stimulatory mechanism might produce additively effects on osteoblast differentiation and bone formation.

Although we need to clarify the signaling crosstalk or the stimulatory mechanisms on bone formation induced by two different ligands, our results clearly showed the beneficial effects of the combination therapy with two different bone forming agents.

Conclusion

The results of this study indicate that W9 peptide can promote bFGF-triggered osteogenesis by stimulating mesenchymal cell proliferation and enhancing early differentiation of osteoblasts *in vitro*. In addition, this peptide also stimulated bFGF-induced bone formation in a rat critical-sized calvarial defect (5 mm in diameter) *in vivo*. Therefore, combination therapy of bFGF and the RANKL-binding peptide W9 could be expected to stimulate bone augmentation at the post-operative bone defect in the maxillofacial region.

Acknowledgments

Disclosure statement

No competing financial interests exist.

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