J Periodont Res 2006; 41; 140–147

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2005.00847.x

Recombinant human growth/differentiation factor-5 (rhGDF-5) induced bone formation in murine calvariae

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Yoshimoto T, Yamamoto M, Kadomatsu H, Sakoda K, Yonamine Y, Izumi Y: Recombinant human growth/differentiation factor-5 (rhGDF-5) induced bone formation in murine calvariae. J Periodont Res 2006; 41: 140–147. © Blackwell Munksgaard 2006

Objectives: Growth/differentiation factor-5 (GDF-5), a member of the transforming growth factor-β superfamily, shows a close structural relationship to bone morphogenetic proteins and plays crucial roles in skeletal morphogenesis. Recombinant human (rh) GDF-5 was reported as a suitable factor for enhancing healing in bone defect and inducing ectopic bone formation. The purpose of the present study was to investigate the mechanism of bone formation induced by rhGDF-5 in murine calvariae by radiological, histological and immunohistochemical methods. Cell proliferation was also examined *in vitro*.

Material and methods: Cells including primary osteoblasts, periosteum cells and connective tissue fibroblasts were isolated enzymatically from neonatal murine calvariae or head skin. In the presence or absence of rhGDF-5, cell proliferation was estimated by tetrazolium reduction assay. To examine the mechanism of osteoinduction, rhGDF-5/atelocollagen (AC) composite or 0.01 N HCl/AC composite were injected into murine calvariae subcutaneously. Tissue was examined radiologically, histologically and immunohistochemically.

Results: In the presence of rhGDF-5, proliferation of primary osteoblasts, periosteum cells, and connective tissue fibroblasts was increased significantly in culture. Immunohistochemical observations showed cells at the site injected with rhGDF-5/AC displayed immunoreactivity for proliferating cell nuclear antigen (PCNA). Newly formed bone- and cartilage-like tissue contained chondrocyte osteocyte and osteoclastic cells, and were immunoreactive for both type I and II collagen.

Conclusion: Exposure to GDF-5 promotes proliferation and differentiation of calvarial cells, which give rise to ectopic bone formation.

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Key words: bone formation; growth factor; immunohistochemical stain; recombinant human growth/ differentiation factor-5

Accepted for publication August 26, 2005

Healing of bone defects is one of the major therapeutic goals in various clinical fields. Heretofore, many filling materials were applied for treatment of alveolar bone defects, such as autograft, allograft, xenograft, and synthetic graft material. One of the successful methods aiming at the treatment of bone defects is autogenous bone graft. However, autografting includes problems such as donor site morbidity and a limited supply, especially in patients with previous graft harvest (1–3). In addition to the application of filling materials, other

methods focusing on activation or enhancement of host cellular functions have been employed for tissue healing and regeneration, including the guided bone regeneration method, enamel matrix derivatives and recombinant growth factors.

Recent studies have centered on the application of osteoinductive recombinant molecules such as bone morphogenetic proteins (BMPs). BMPs are known to induce *de novo* bone formation. These proteins are involved in cascades regulating cellular events, such as stem-cell commitment, proliferation, and differentiation in tissue formation and regeneration. Either recombinant human osteogenic protein-1 or recombinant human (rh) BMP-2 combined with type-I collagen sponge have been found to elicit healing of segmental bone defects and periodontal defects in animal models (4–10). However, some studies have reported the occurrence of unfavorable events during healing, such as root resorption and ankylosis after implantation of rhBMP-2 (4, 9, 10). This aberrant healing in response to exogenous BMP-2 must be addressed before clinical trials can commence.

Growth/differentiation factor-5 (GDF-5), also known as cartilage-derived morphogenetic protein-1, shows a close structural relationship to BMPs. GDF-5 is also a member of the transforming growth factor-β superfamily, sharing BMP receptor type IB with BMP-2/4 (11). GDF-5 is considered to play a crucial role in skeletal morphogenesis based on the following biological observations. Mice with brachypodism, which reduced limb size, were found to have a missense mutation in the GDF-5 gene (12–17). Growth/differentiation factor-5 promotes mesenchymal cell recruitment and skeletal processes such as endochondral ossification, synovial joint formation, tendon/ligament development and odontogenesis (18–24). A lot of *in vitro* experiments suggested that GDF-5 enhanced chondrogenic or osteogenic differentiation in mesenchymal cell cultures via the BMP receptor-Smads signal pathway (25–28). Recombinant GDF-5 protein administration with suitable carriers induced formation of tendon/ligament-like, cartilage-like and/or bone-like tissues in some rodents, ruminants and primates (15, 29–31). However, the mechanism of bone induction by GDF-5 in craniofacial tissue remained to be identified.

In the present study, we investigated mechanisms of osteogenic induction by rhGDF-5 in murine calvariae using radiological, histological and immunohistochemical methods. Cell proliferation was also examined *in vitro*.

Material and methods

The rhGDF-5 protein was courtesy of Biopharm GmbH (Heidelberg, Germany). This protein was dissolved in 0.01 N HCl (1.0 μg/μl) and stored at −80°C until use.

Cell culture and measurement of cell proliferation

Calvariae-derived cells such as primary osteoblasts and periosteum cells and connective tissue fibroblasts of the scalp were enzymatically isolated from dissected neonatal murine calvariae and avulsed head skin (32). They were seeded in culture plates at cell densities specified in the figure legends, and cultured overnight in α-minimal essential medium (MEM) supplemented with 10% fetal bovine serum. The medium was subsequently replaced with α-MEM supplemented with 1% fetal bovine serum and various concentrations of rhGDF-5 ranging from 0 to 500 ng/ml. Cells were cultured for periods as specified in the figure legends, with substitution of fresh medium every 3 days. Cell proliferation was evaluated using a kit employing a tetrazolium salt that produces a highly water-soluble formazan dye agents (Cell-counting kit-8®; Dojindo Laboratories, Kumamoto, Japan). After 1 h of incubation with the reagent, relative cell number was estimated by measuring light absorbance at a wavelength of 450 nm by formazan dye products in the cultures.

Injection of recombinant human growth/differentiation factor-5

Atelocollagen (AC) solution derived from porcine skin (3 mg/ml, pH 3.0, Cellmatrix I-A®, Nitta Gelatin, Inc., Osaka, Japan) was used as a carrier. Recombinant human growth/differentiation factor-5 (20 μg) was mixed with AC solution (20 μl) and neutralized to pH 7.4. As a control, 0.01 N HCl (20 μl) was mixed with AC solution (20 μl). Then 8-week-old ddy mice ($n = 4$ per experimental condition and time point) were anesthetized by intra-abdominal injection of sodium pentobarbital (5 mg/kg Nembutal Injection®; Dainippon Pharmaceutical Co., Ltd.). Subsequently, either rhGDF-5/AC composite or 0.01 N HCl/AC composite, was injected by Tuberculin syringe® (Terumo Medical Corporation, Tokyo, Japan) beneath the scalp along the periosteum region.

Tissue preparation

For light microscopic examination, mice were killed 3, 7, 14, 21, or 28 days after the injection. Tissue blocks, including calvariae and surrounding soft tissue, were dissected out and fixed in 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4) for 24 h at 4°C. Specimens were demineralized with 0.5 M ethylenediaminetetraacetic acid (EDTA; pH 7.4) for 3 weeks at 4°C. After dehydration through a graded series of ethanol solutions, specimens were embedded in paraffin. Serial sections 6-μm thick were cut with a microtome and affixed onto glass slides.

Histology

After deparaffinization with xylene and ethanol, sections were stained with hematoxylin and eosin. For detection of non-collagenous matrices, alcian blue staining was performed.

Immunohistochemistry

Immunohistochemical staining was performed to detect type I and II collagen, proliferating cell nuclear antigen (PCNA) and tartrate-resistant acid phosphatase (TRAP) in sections. After deparaffinization, sections were immersed in phosphate-buffered saline (PBS). Before detection of type I and II collagen, sections were digested with testicular hyaluronidase (Sigma

Chemicals, St Louis, MO, USA), 25 mg/ml in PBS for 30 min at 37°C. After several washes in PBS, the sections were immersed in methanol containing 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. After several additional washes in PBS, the sections were immersed in 10% normal goat serum (Dako Corporation, Carpinteria, CA, USA) to block non-specific reactions. Sections were subsequently incubated overnight at 4°C with rabbit polyclonal antibody against type I collagen (Biogenesis, Poole, UK) diluted at 1:200 with PBS containing 2% normal goat serum, rabbit polyclonal antibody against type II collagens (LSL, Tokyo, Japan) diluted at 1:1000 with PBS containing 2% normal goat serum (33), anti-PCNA antibody (Dako Corporation) diluted at 1:100 with PBS (34), or anti-TRAP antibody (Laboratory Vision Corporation, Fremont, CA, USA) diluted at 1:20 with PBS. After several washings in PBS, sections were incubated with biotin-labeled secondary antibodies (Nichirei Corporation, Tokyo, Japan). After several washes in PBS, sections were incubated with peroxidase-labeled streptavidin. After several more washes in PBS, sections were treated with a 3,3′-diaminobenzidine solution to visualize the reaction and then inspected after counterstaining with hematoxylin. As negative controls, sections were incubated with normal rabbit immunoglobulin fraction or mouse negative control IgG_{2a} or IgG_{2b} (Dako Corporation) instead of the primary antibodies.

Micro-computed tomography

At 14 days after the injection, calvariae were analyzed using a micro-computed tomography system (MCT-CB100MFZ®, Hitachi Medical Corporation, Tokyo, Japan). Newly formed bone was imaged with 201 slices using a cone-beam type scan. Architecture of the calvariae was reconstructed three-dimensionally with software (Micro CT Pro, Kubota Graphics Technologies Co., Ltd, Tokyo, Japan) at a threshold of 1200 to eliminate soft tissue data from the bone data.

Statistical analysis

Data are expressed as the mean ± standard deviation. Statistical significance of differences between groups was assessed by one-way analyses of variance (ANOVA) and *post hoc* *t*-tests.

Results

Formation of bone-like tissue induced by recombinant human growth/differentiation factor-5

In our preliminary studies on murine calvariae, the observed increase of newly formed bone volume was in accordance with the rhGDF-5 dose, adjusting to 0 μg, 0.2 μg, 2.0 μg, and 20 μg (data not shown). The dose of rhGDF-5 in the present study was set at 20 μg. At 14 days after the injection, newly formed bone-like tissue was observed histologically (Figs 1a and b) and by using micro-computed tomography (Figs 1c and d) on the calvariae. No abnormalities in general condition or weight were observed in either rhGDF-5/AC or HCl/AC mice over the experimental period. At 3 days after the injection, thickened tissue was observed where rhGDF-5/AC had been injected. Microscopically, mesenchymal cells were confirmed to be present in the region of the rhGDF-5/AC injection. Some of these cells were immunoreactive for PCNA immunohistochemical staining (Fig. 2a, 3 days). No histological change was observed in the HCl/AC group (data not shown). At 7 days after the injection, mesenchymal cells in the region of rhGDF-5/AC injection were immunoreactive for PCNA (Fig. 2a, 7 days). At 14 days after the injection, some of cells in the periosteum area showed an immunoreaction for PCNA (Fig. 2a, 14 days).

To determine whether exogenous rhGDF-5 affected proliferation of primary osteoblasts, periosteum cells or connective tissue fibroblasts cells, cell numbers in a culture system were estimated indirectly using formazan dye at various time points. After 3 and 9 days in culture in the presence of rhGDF-5, cell numbers had increased significantly compared with those of cells unexposed to rhGDF-5 (Fig. 2b). Although the decrease was observed in connective tissue fibroblasts cells, it was obvious that rhGDF-5 increased connective tissue fibroblasts cell proliferation at each respective point in time. At this point, the result was similar to our previous study concerning human periodontal ligament cells (35).

Characteristics of newly formed bone-like tissue

At 14 and 21 days after the injection, formation of newly formed bone/cartilage-like tissue was evident on hematoxylin and eosin-stained sections from rhGDF-5/AC-treated mice (Figs 3c and d). These tissues show an apparent bone marrow-like structure. Alcian blue staining indicated that these tissues contained acid mucopolysaccharide (Figs 3h and i), and chondrocyte-like cells and osteocyte-like cells were also observed in these tissues. Chondrocyte-like cells were surrounded with tissues strongly stained for alcian blue (Figs 3h and i). These tissues showed immunoreactivity for both type I and type II collagens (Figs 3m, n, r and s). At 14 and 21 days after the injection, newly formed bone/cartilage-like tissue was strongly positive for type-II collagen and alcian blue staining, which are relevant to cartilage.

At 28 days after the injection, newly formed bone/cartilage-like tissue was detected in hematoxylin and eosin-stained sections from rhGDF-5/AC mice (Fig. 3e), and osteocyte-like cells were observed in these tissues. Bone marrow-like areas were also observed. TRAP-positive multinucleated cells were detected in the center of these bone marrow-like areas (Fig. 4). Moreover, smaller TRAP-positive cells containing three to five nuclear and TRAP-positive mononuclear cells were located adjacent to the cell matrix.

These tissues were strongly immunostained for type I collagen, as were areas surrounding osteocyte-like cells (Fig. 3o). At 28 days after the injection, newly formed bone/cartilage-like

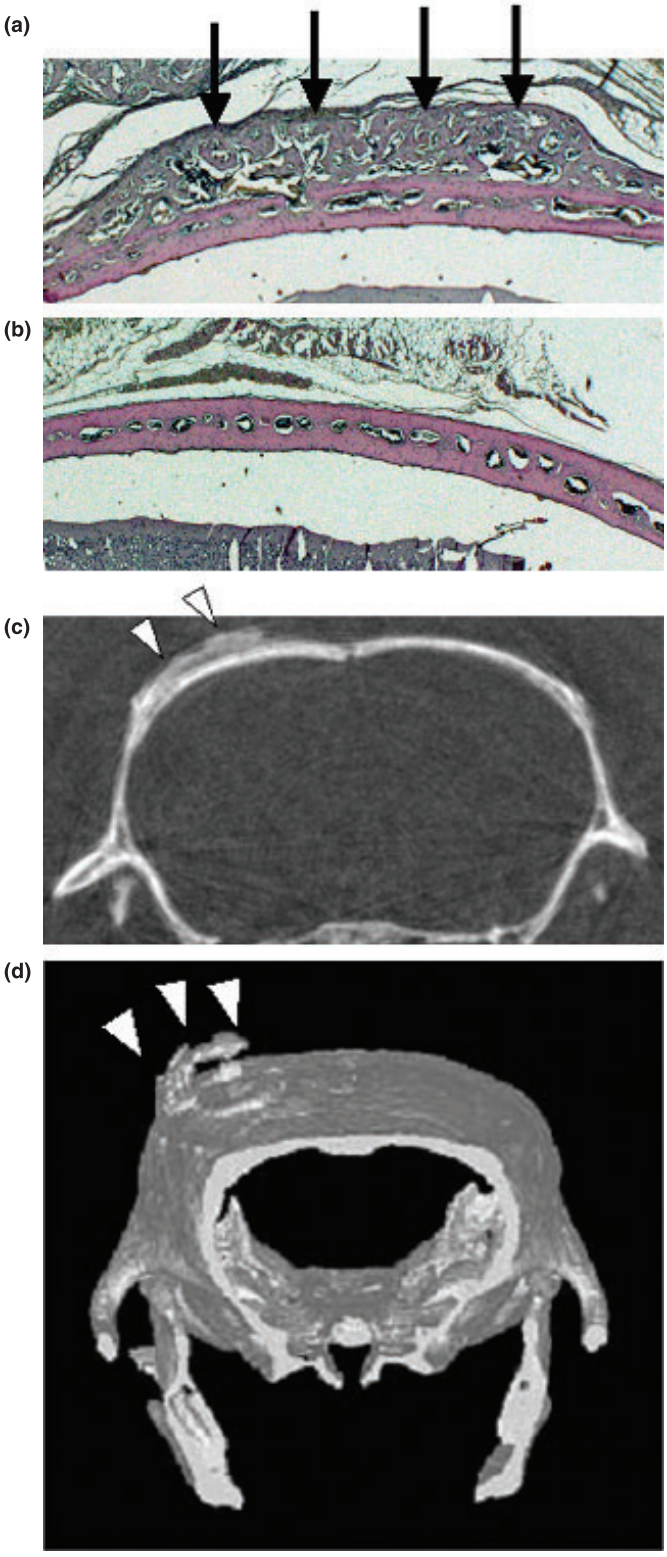

Fig. 1. At 14 days after the injection, newly formed bone-like tissue was observed in murine calvarial sections stained with hematoxylin and eosin (arrow); original magnification ×12. Micro-computed tomography showed a newly formed bone-like radiopaque region upon calvariae (arrowhead). (a) Recombinant human growth/differentiation factor-5 (rhGDF-5) injected region; (b) non-treated region; (c) two-dimensional micro-computed tomography; (d) three-dimensional micro-computed tomography.

tissue was strongly positive for type-I collagen, which is relevant to bone.

Discussion

Two types of process contribute to bone development, namely intramembranous and endochondral ossification. In the present study, we focused on mechanisms of endochondral ossification, although calvariae would have undergone intramembranous ossification in normal development. For ectopic bone formation, it is necessary that cells migrate into the area, proliferate and produce extracellular matrices. GDF-5 has been reported to promote mesenchymal cell aggregations in a dose-dependent manner (36, 37). In our murine experiments, cell migration could have occurred after injection because the rhGDF-5/AC injection was free of cells at the beginning of the experiment. We found that exogenous rhGDF-5 stimulated proliferation of primary osteoblasts, periosteum cells and connective tissue fibroblasts cells *in vitro*, especially in the latter two (Fig. 2b). Our immunohistochemical analysis also showed that cells in the torus are immunoreactive for PCNA, a DNA polymerase-associated nuclear protein involved in DNA synthesis in mitosis (Fig. 2a, 3 days, 7 days and 14 days). We therefore believe that cells migrated from calvarial periphery tissue into the injected rhGDF-5/AC composite. Overall, it is thought that formation of bone-like tissue in the area injected with rhGDF-5/AC composite is due to the migration and proliferation of peripheral cells, especially periosteum cells and connective tissue fibroblasts, induced by rhGDF-5 (Figs 3a, b, f, g, k, l, p and q).

In general, AC acts as a scaffold for cell migration. However, few cells were observed within AC at 3 or 7 days after injection, and no torus could be confirmed histologically at 14–28 days after injection of HCl/AC. It is therefore suggested that the period of HCl/AC remaining inside the tissue was insufficient for migration and proliferation of peripheral cells as well as for producing an extracellular matrix. It was reported that rhGDF-5 with

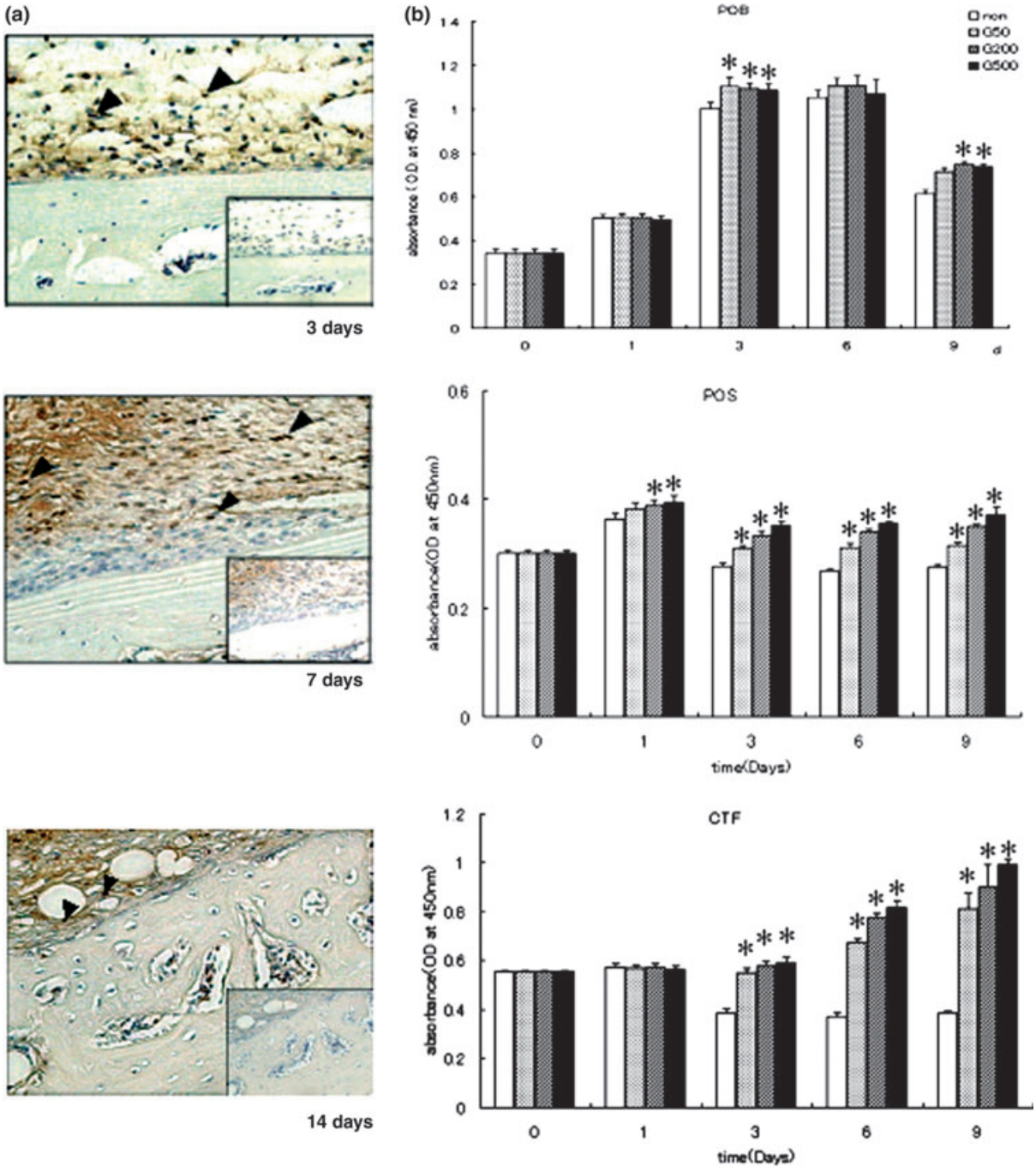

Fig. 2. Effect of recombinant human growth/differentiation factor-5 (rhGDF-5) on murine cell proliferation. (a) Anti-proliferating cell nuclear antigen (PCNA) immunohistochemical staining at 3, 7 and 14 days after the injection; original magnification ×12. Inset shows a negative control with non-immune IgG substitute for primary antibody. At 3, 7 and 14 days after the injection, PCNA-immunoreactive cells were seen at the injection site (arrowhead). (b) Cells were seeded at a density of 5×10^3 cells/cm^2 in a 24-well culture plate and incubated for 1 day. Subsequently, cells were treated with rhGDF-5 at concentrations of 0, 50, 200 and 500 ng/ml for 1, 3, 6 and 9 days. Error bars indicate the standard deviation of the mean of measurements in three wells. *Significantly different from control ($p < 0.01$).

absorbable collagen sponge induced bone augmentation in rat calvariae (31). Similarly, we found that a single injection of rhGDF-5/AC induced bone formation on murine calvariae. Heidaran *et al.* (38) recently reported that GDF-5 increases synthesis of extracellular matrix, such as proteoglycan, aggrecan and type-II collagen associated with chondrogenesis *in vitro*. Previous experiments in our laboratory suggested that rhGDF-5 stimulates proliferation and sulphated glycosaminoglycan (sGAG) synthesis in primary human periodontal liga-

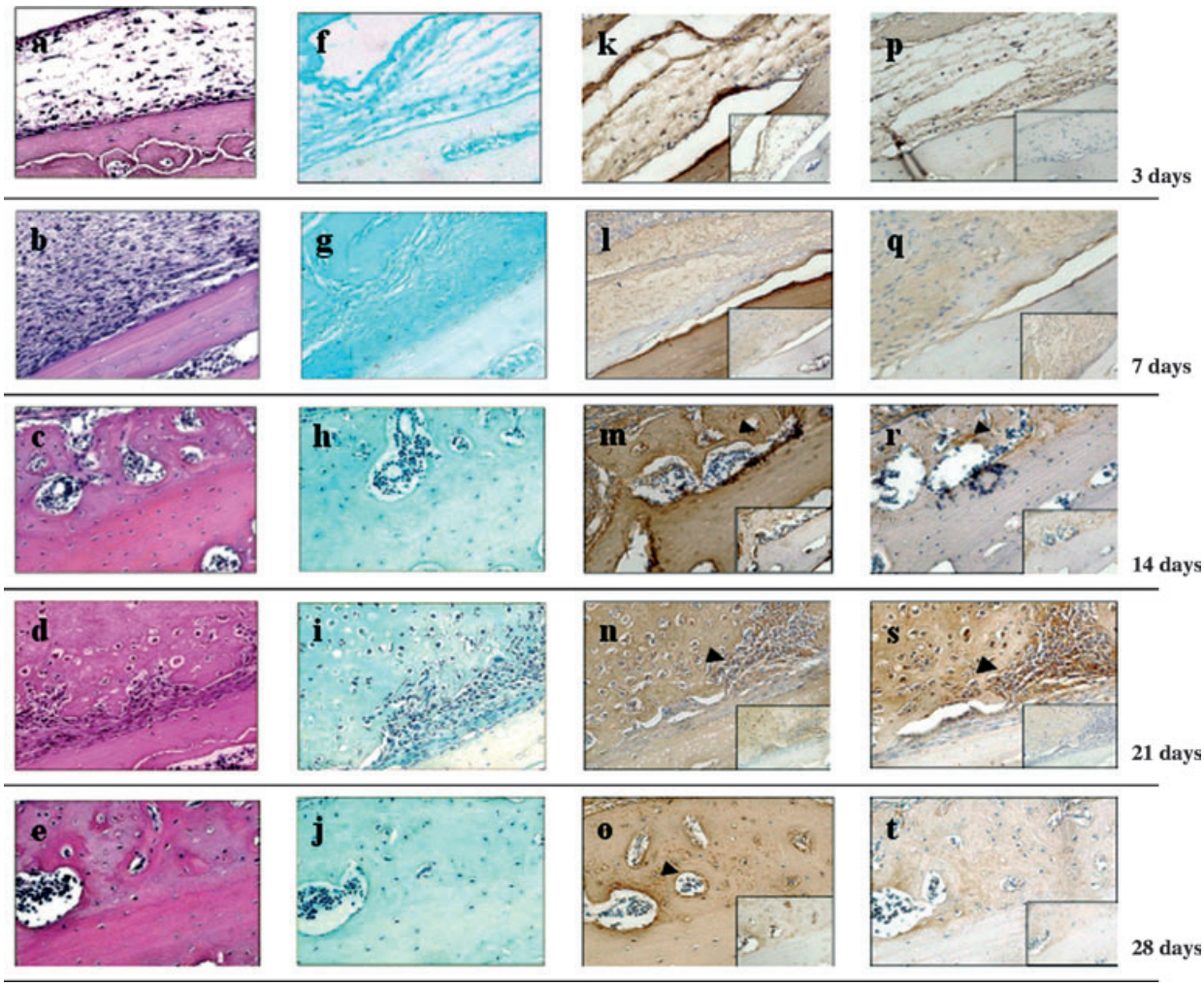

Fig. 3. Light micrographs of the recombinant human growth/differentiation factor-5 (rhGDF-5)/atelocollagen (AC) injected area during ectopic bone formation; original magnification ×12. Stains shown are hematoxylin and eosin (a–e), alcian blue (f–j), anti-type-I collagen immunohistochemical staining (collagen-I, k–o) and anti-type-II collagen immunohistochemical staining (collagen-II, p–t). Times shown are 3 days after injection (a, f, k, p), 7 days after injection (b, g, l, q), 14 days after injection (c, h, m, r), 21 days after injection (d, i, n, s) and 28 days after injection (e, j, o, t). Inset shows a negative control stained with non-immune IgG substitute for primary antibody. Newly formed bone was strongly stained by alcian blue. These tissues were positive for type I collagen (m–o) and type II collagen (r–t). Co-expression of types I and II collagens was observed (arrowhead).

ment cells (35). In the present study, newly formed bone/cartilage-like tissue was observed in the rhGDF-5/AC injected area. These tissues were strongly stained by alcian blue, suggesting acid mucopolysaccharide is strongly accumulated in these tissues (Figs 3h–j). Moreover, these tissues showed immunoreactivity for antitype-II collagen. These findings strongly suggest that rhGDF-5 induced ectopic bone formation via endochondral ossification. In skeletal development, GDF-5 is considered to promote mesenchymal cell recruitment and skeletal processes such as endochondral ossification, and also odontogenesis (19). Healing was enhanced in the rat tendon rupture model with a suture immersed with rhGDF-5 (15). Bone defects in tibiae in baboons were regenerated with rhGDF-5/atero-collagen sponges in a dose-dependent manner (39). These observations encourage us to promote research and development of regenerative therapy using rhGDF-5 for bone and periodontal diseases.

Angiogenesis is indispensable in bone formation. According to studies on the angiogenic activity of the BMP family, GDF-5 induced angiogenesis in both chick chorioallantoic membranes and rabbit cornea assays, although BMP-2 did not (40). This angiogenic activity of GDF-5 was probably involved in the replacement of ectopic cartilage-like tissue with bone-like tissue. Although BMP-2 has been reported to induce ectopic bone formation, its vascular inductive potency has not been clearly demonstrated.

Figure 4 shows multinucleated cells in a bone marrow-like space of the newly formed bone. Moreover,

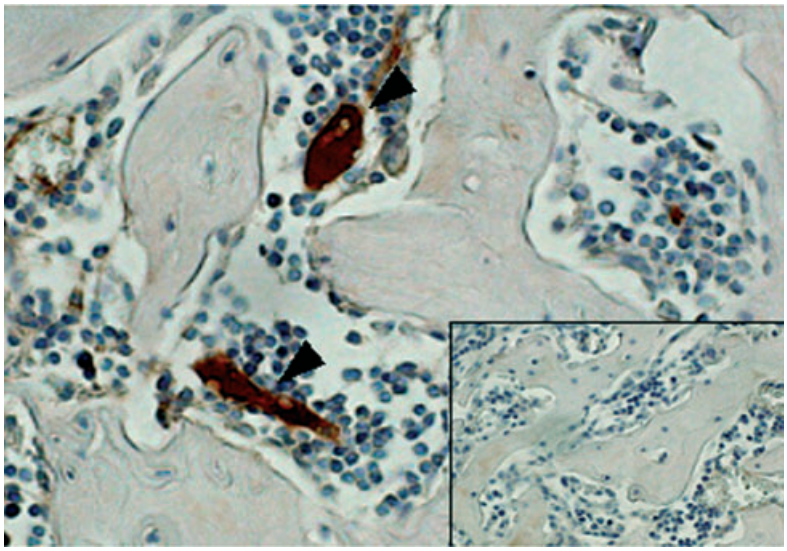

Fig. 4. Anti-tartrate-resistant acid phosphatase (TRAP) immunohistochemical staining. Inset shows a negative control with non-immune IgG substitute for primary antibody; original magnification ×30. TRAP-positive multinuclear cells were observed in a newly formed structure resembling bone marrow (arrowhead).

TRAP-positive cells containing three to five nuclear and smaller TRAP-positive mononuclear cells were located adjacent to the cell matrix. These TRAP-positive mononuclear cells were activated to fuse with each other into a multinucleated cell. Figure 4 indicates that active osteoclastogenesis has occurred in the bone marrow-like tissues.

During endochondral bone formation, there are proliferating chondrocytes in the epiphysial cartilage. Proliferating chondrocytes differentiate to hypertrophic chondrocytes, which promotes vascular invasion supplying chondroclast/osteoclasts resulting in cavity formation. As a result, woven bone is formed beneath the epiphysial plate. Just after calcification of this woven bone, osteoclasts and subsequently blood vessels penetrate the cartilage, carrying the blood supply that will form the hematopoietic bone marrow (41). Progenitors related to osteoblasts are also recruited and produce bone matrix through blood vessels. As the result of these processes, endochondral ossification has occurred in the epiphysial plate. The existence of TRAP-positive multinucleated cells suggests development of vascular and metabolic features of bone in the ectopic bone induced by rhGDF-5/AC (Fig. 4).

In conclusion, we found that rhGDF-5 stimulated calvarial cell migration, proliferation, and extracellular matrix production. A composite of rhGDF-5/AC was effective in inducing ectopic bone formation in a way similar to endochondral ossification.

Acknowledgements

We thank Dr Hideki Sekiya (First Department of Oral Surgery) and Drs Kenzo Kawasaki and Shinji Shimoda (First Department of Anatomy) at Tsurumi University for their assistance in micro-computed tomography analysis, as well as Dr Jens Pohl (BIOPHARM GmbH) for kindly providing the rhGDF-5.

References

1. Goldberg VM, Stevenson S. Natural history of autografts and allografts. *Clin Orthop* 1987;225:7–16.
2. Springfield D. Autograft reconstructions. *Orthop Clin North Am* 1996;**27:**483–492.
3. Fennis JP, Stoelinga PJ, Jansen JA. Reconstruction of the mandible with an autogenous irradiated cortical scaffold, autogenous corticocancellous bone-graft and autogenous platelet-rich-plasma: an animal experiment. *Int J Oral Maxillofac Surg* 2005;**34:**158–166.
4. Wikesjo UM, Guglielmoni P, Promsudthi A *et al*. Periodontal repair in dogs: effect of rhBMP-2 concentration on regeneration of alveolar bone and periodontal attachment. *J Clin Periodontol* 1999;**26:**392–400.
5. Cook SD, Baffes GC, Wolfe MW, Sampath TK, Rueger DC. Recombinant human bone morphogenetic protein-7 induces healing in a canine long-bone segmental defect model. *Clin Orthop* 1994;301: 302–312.
6. Cook SD, Salkeld SL, Brinker MR, Wolfe MW, Rueger DC. Use of an osteoinductive biomaterial (rhOP-1) in healing large segmental bone defects. *J Orthop Trauma* 1998;**12:**407–412.
7. Giannobile WV, Ryan S, Shih MS *et al*. Recombinant human osteogenic protein-1 (OP-1) stimulates periodontal wound healing in class III furcation defects. *J Periodontol* 1998;**69:**129–137.
8. Blumenthal NM, Koh-Kunst G, Alves ME *et al*. Effect of surgical implantation of recombinant human bone morphogenetic protein-2 in a bioabsorbable collagen sponge or calcium phosphate putty carrier in intrabony periodontal defects in the baboon. *J Periodontol* 2002;**73:**1494–1506.
9. Selvig KA, Sorensen RG, Wozney JM, Wikesjo UM. Bone repair following recombinant human bone morphogenetic protein-2 stimulated periodontal regeneration. *J Periodontol* 2002;**73:**1020–1029.
10. King GN, Hughes FJ. Effects of occlusal loading on ankylosis, bone, and cementum formation during bone morphogenetic protein-2-stimulated periodontal regeneration *in vivo*. *J Periodontol* 1999;**70:**1125–1135.
11. Nishitoh H. [Identification of receptors for bone morphogenetic proteins]. *Kokubyo Gakkai Zasshi* 1997;**64:**24–37.
12. Storm EE, Huynh TV, Copeland NG *et al*. Limb alterations in brachypodism mice due to mutations in a new member of the TGF beta-superfamily. *Nature* 1994;**368:**639–643.
13. Mikic B, Schalet BJ, Clark RT, Gaschen V, Hunziker EB. GDF-5 deficiency in mice alters the ultrastructure, mechanical properties and composition of the Achilles tendon. *J Orthop Res* 2001;**19:**365–371.
14. Clark RT, Johnson TL, Schalet BJ *et al*. GDF-5 deficiency in mice leads to disruption of tail tendon form and function. *Connect Tissue Res* 2001;**42:**175–186.
15. Wolfman NM, Hattersley G, Cox K *et al*. Ectopic induction of tendon and ligament in rats by growth and differentiation factors 5, 6, and 7, members of the TGF–beta gene family. *J Clin Invest* 1997;**100:**321–330.
16. Storm EE, Kingsley DM. Joint patterning defects caused by single and double

mutations in members of the bone morphogenetic protein (BMP) family. *Development* 1996;**122:**3969–3979.
17. Mikic B, Battaglia TC, Taylor EA, Clark RT. The effect of growth/differentiation factor-5 deficiency on femoral composition and mechanical behavior in mice. *Bone* 2002;**30:**733–737.
18. Morotome Y, Goseki-Sone M, Ishikawa I, Oida S. Gene expression of growth and differentiation factors-5, -6, and -7 in developing bovine tooth at the root forming stage. *Biochem Biophys Res Commun* 1998;**244:**85–90.
19. Sena K, Morotome Y, Baba O *et al*. Gene expression of growth differentiation factors in the developing periodontium of rat molars. *J Dent Res* 2003;**82:**166–171.
20. Buxton P, Edwards C, Archer CW, Francis-West P. Growth/differentiation factor-5 (GDF-5) and skeletal development. *J Bone Joint Surg Am* 2001;**83-A** (supplement 1)**:**S23–S30.
21. Archer CW, Dowthwaite GP, Francis-West P. Development of synovial joints. *Birth Defects Res Part C Embryo Today* 2003;**69:**144–155.
22. Settle SH Jr, Rountree RB, Sinha A *et al*. Multiple joint and skeletal patterning defects caused by single and double mutations in the mouse Gdf6 and Gdf5 genes. *Dev Biol* 2003;**254:**116–130.
23. Edwards CJ, Francis-West PH. Bone morphogenetic proteins in the development and healing of synovial joints. *Semin Arthritis Rheum* 2001;**31:**33–42.
24. Coleman CM, Tuan RS. Functional role of growth/differentiation factor 5 in chondrogenesis of limb mesenchymal cells. *Mech Dev* 2003;**120:**823–836.
25. Nishitoh H, Ichijo H, Kimura M *et al*. Identification of type I and type II serine/threonine kinase receptors for growth/differentiation factor-5. *J Biol Chem* 1996;**271:**21345–21352.
26. Baur ST, Mai JJ, Dymecki SM. Combinatorial signaling through BMP receptor IB and GDF5: shaping of the distal mouse limb and the genetics of distal limb diversity. *Development* 2000;**127:**605–619.
27. Akiyama H, Shukunami C, Nakamura T, Hiraki Y. Differential expressions of BMP family genes during chondrogenic differentiation of mouse ATDC5 cells. *Cell Struct Funct* 2000;**25:**195–204.
28. Aoki H, Fujii M, Imamura T *et al*. Synergistic effects of different bone morphogenetic protein type I receptors on alkaline phosphatase induction. *J Cell Sci* 2001;**114:**1483–1489.
29. Simank HG, Manggold J, Sebald W *et al*. Bone morphogenetic protein-2 and growth and differentiation factor-5 enhance the healing of necrotic bone in a sheep model. *Growth Factors* 2001;**19:**247–257.
30. Simank HG, Sergi C, Jung M *et al*. Effects of local application of growth and differentiation factor-5 (GDF-5) in a full-thickness cartilage defect model. *Growth Factors* 2004;**22:**35–43.
31. Kuniyasu H, Hirose Y, Ochi M *et al*. Bone augmentation using rhGDF-5-collagen composite. *Clin Oral Implants Res* 2003;**14:**490–499.
32. Partridge NC, Alcorn D, Michelangeli VP *et al*. Functional properties of hormonally responsive cultured normal and malignant rat osteoblastic cells. *Endocrinology* 1981; **108:**213–219.
33. Shibata S, Fukada K, Suzuki S, Yamashita Y. Immunohistochemistry of collagen types II and X, and enzyme-histochemistry of alkaline phosphatase in the developing condylar cartilage of the fetal mouse mandible. *J Anat* 1997;**191:** 561–570.
34. Li G, White G, Connolly C, Marsh D. Cell proliferation and apoptosis during fracture healing. *J Bone Miner Res* 2002;**17:**791–799.
35. Nakamura T, Yamamoto M, Tamura M, Izumi Y. Effects of growth/differentiation factor-5 on human periodontal ligament cells. *J Periodontal Res* 2003;**38:**597–605.
36. Francis-West PH, Abdelfattah A, Chen P *et al*. Mechanisms of GDF-5 action during skeletal development. *Development* 1999;**126:**1305–1315.
37. Hatakeyama Y, Tuan RS, Shum L. Distinct functions of BMP4 and GDF5 in the regulation of chondrogenesis. *J Cell Biochem* 2004;**91:**1204–1217.
38. Heidaran M, Daverman R, Thompson A *et al*. Extracellular Matrix Modulation of rhGDF-5-Induced Cellular Differentiation. *e-biomed* 2001;**1:**121–135.
39. Spiro RC, Liu L, Heidaran MA *et al*. Inductive activity of recombinant human growth and differentiation factor-5. *Biochem Soc Trans* 2000;**28:**362–368.
40. Yamashita H, Shimizu A, Kato M *et al*. Growth/differentiation factor-5 induces angiogenesis *in vivo*. *Exp Cell Res* 1997;**235:**218–226.
41. Adams JS, Baran DT, Baron R. *et al*. Chapter 1 In: Favus MJ, ed. *Primer on the metabolic bone diseases and disorders of mineral metabolism*, 4th edition. Lippincott: Williams & Wilkins, 1999: 3–10.
42. Niida S, Kaku M, Amano H *et al*. Vascular endothelial growth factor can substitute for macrophage colony-stimulating factor in the support of osteoclastic bone resorption. *J Exp Med* 1999;**190:** 293–298.