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Injectable growth/differentiation factor-5–recombinant human collagen composite induces endochondral ossification via Sry-related HMG box 9 (Sox9) expression and angiogenesis in murine calvariae

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Background and Objective: The types of collagens available today as biomaterials are purified from animal tissues. A major growing concern, however, is their safety, since there are risks of viral and prion contamination and of unknown and potentially zoonotic infectious diseases. The present study aimed to assess, using immunohistochemistry, the effects of recombinant human growth/differentiation factor-5 (rhGDF-5) combined with recombinant human collagen I (rhCI) on bone formation in murine calvariae.

Material and Methods: Composite rhGDF-5–rhCI or rhCI alone was injected subcutaneously into murine calvariae. After 3, 7 or 14 days, tissues were examined radiologically, histologically and immunohistochemically. The production of vascular endothelial growth factor (VEGF) by primary osteoblasts, periosteal cells and connective tissue fibroblasts isolated enzymatically from neonatal murine calvariae was also assessed.

Results: A protrusion was observed on the calvariae at the site injected with rhGDF-5/rhCI composite. Its mineral density was shown to be different from that of the existing bone by two-dimensional microcomputed tomography. Type II collagen-positive staining was restricted to newly formed tissues. Thus, the newly formed tissues seemed to be bone- and cartilage-like tissues. A number of vessels with positively stained cells for Von Willebrand factor were detected in the newly formed tissues. The rhGDF-5 enhanced VEGF production in cultured connective tissue fibroblasts. Sry-related HMG box 9 (Sox9)-positive

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cells were detected in the hypertrophic periosteum, and penetrated into the newly formed tissues.

Conclusions: These results suggest that rhCI seems to allow the release of rhGDF-5 and that rhGDF-5–rhCI composite induces endochondral ossification via Sox9 expression and angiogenesis in murine calvariae.

Collagen is biocompatible and degradable and has been used for the delivery of a variety of proteins, including vascular endothelial growth factor (VEGF), fibroblast growth factor and heparin (1,2). Atelocollagen is produced by telopeptide removal from natural collagen molecules, resulting in extremely low antigenicity. However, the use of bovine collagen should be reconsidered and restricted because of the risks of bovine spongiform encephalopathy, which is considered to be transmittable to humans. Therefore, recombinant collagens have been developed using transfected mammalian cells (3,4), insect cells, yeast (5,6) and *Escherichia coli* (7). Among these alternatives, recombinant human collagen obtained from strains of the yeast *Pichia pastoris* is derived from mostly monomeric collagen or fragments of a single chain compared to those of animal tissues, and involves the generation of single triple-helical molecules that are then used to construct more complex three-dimensional structures (8). This allows the delivery of a drug for tissue engineering without the disadvantages of animal-tissue-derived collagen.

Recent studies have focused on the applications of osteoconductive recombinant molecules, such as bone morphogenetic proteins (BMPs). In addition, the application of BMP-2 combined with type I collagen has been reported to induce healing of bone defects and periodontal defects in animal models (9–12). Some studies, however, have reported unfavourable events during healing, including root resorption and ankylosis following application of recombinant human BMP-2 in periodontal defects (9,11,12). This aberrant healing response to ossification needs to be resolved prior to clinical trials. In addition, the application of other recombinant factors using type I collagen would be necessary for normal healing in periodontal defects.

Growth/differentiation factor-5 (GDF-5), also known as cartilage-derived morphogenetic protein-1, is closely related to bone morphogenetic proteins (13,14). It binds with high affinity to the BMP type Ib receptor, which is activated upon recruitment of the BMP type II receptor, and is considered to play a crucial role in skeletal morphogenesis. For example, GDF-5 promotes mesenchymal cell recruitment and skeletal processes such as endochondral ossification, synovial joint formation, tendon/ligament development and odontogenesis (15–21). The administration of recombinant GDF-5 with suitable carriers also induces the formation of tendon-/ligament-like and cartilage-/bone-like tissues in some rodents, ruminants and primates (22–24). We previously showed that rhGDF-5 administration with porcine atelocollagen solution induced ectopic bone formation in murine calvariae and promoted the proliferation of calvarial cells (25). The mechanisms involved in the differentiation of calvarial cells in response to rhGDF-5 administration in murine calvarie remain unclear, however.

In the present study, we assessed the effects of recombinant human collagen I (rhCI), for the controlled release of rhGDF-5, on bone formation in murine calvariae. We examined the effects of rhGDF-5 administration on the differentiation of calvarial cells using immunohistochemistry.

Material and methods

Injection of rhGDF-5

Recombinant Human Collagen (rhCI; FibroGen Inc., South San Francisco, CA, USA) was used as carrier. In addition, rhGDF-5 (20 μg; BIOPHARM GmbH, Heidelberg, Germany) was mixed with rhCI solution (20 μl) as test. As control, 0.01 N HCl neutralized to pH 7.4 was mixed with rhCI solution (20 μl). Eight-week-old ddY mice ($n = 30$) were anaesthetized by intra-abdominal injection of sodium pentobarbitone (5 mg/kg Nembutal Injection®; Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan) and rhGDF-5/rhCI composite or 0.01 N HCl/rhCI composite was injected using a Tuberculin syringe® (Terumo Medical Corporation, Tokyo, Japan) subcutaneously beneath the scalp, along the periosteal region.

Tissue preparation

Mice were killed at 3, 7 or 14 days after injection. Tissue blocks including calvariae and surrounding soft tissues were dissected out and fixed in 4% paraformaldehyde in 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 21 days at 4°C. After dehydration through a graded series of ethanol solutions, specimens were embedded in paraffin. Serial sections (thickness, 6 μm) were cut with a microtome and affixed onto glass slides for light microscopic examination.

Histology

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

Immunohistochemistry

Immunohistochemical staining was performed to detect type I and II collagens, Von Willebrand Factor (VWF)

and Sry-related HMG box 9 (Sox9). Following deparaffinization, sections were immersed in phosphate-buffered saline (PBS). After several washes in PBS, sections were immersed in methanol containing 0.3% hydrogen peroxide for 30 min to block endogenous peroxidase activity. After several additional washes in PBS, sections were immersed in DAKO Protein Block Serum Free (Dako Corp., Carpinteria, CA, USA) to block non-specific reactions. Sections were subsequently incubated for 1 h at room temperature with purified rabbit antimouse type I collagen (Cedarlanelabs, Hornby, Ontario, Canada) diluted at 1:250 with DAKO Cytomation antibody diluent, purified rabbit antimouse type II collagen (Cedarlanelabs) diluted at 1:200, VWF (Dako) diluted at 1:200 or Sox9 (CHEMICON International Inc., Temecula, CA, USA) diluted at 1:100. After several further washes in PBS, sections were incubated with biotin-labelled secondary antibodies, followed by peroxidase-labelled streptavidin, and finally treated with a 3,3′-diaminobenzidine solution to visualize the reaction (Dako Cytomation LSAB + System HRP). Each incubation step was followed by washes in PBS. Sections were inspected after counterstaining with haematoxylin.

As negative controls, sections were incubated with normal goat IgG (Peprotech EC, London, UK) or normal rabbit negative control IgG (Dako Corp.) instead of the primary antibodies.

Microcomputed tomography

Fourteen days after injection, calvariae were analysed using a microcomputed tomography system (Scan Xmate-E080®, Comscantecno Co., Ltd, Kanagawa, Japan). The calvaria was fixed vertically a stage and the sensor of tomography was squarely pointed for the measurement area.

Newly formed bone was imaged with 201 slices using a scan.

Cell culture and VEGF enzyme-linked immunosorbent assay

Primary osteoblasts, periosteal cells and connective tissue fibroblasts enzymatically isolated from dissected neonatal murine calvariae and avulsed head skin were used in the present study (25). Each cell type was grown in α-minimal essential medium (MEM) supplemented with 10% fetal bovine serum. Cells (5×10^4 cells per well) were seeded into 96-well plates, in the presence or absence of rhGDF-5 (1 ng/ml) for 24 h with α-MEM supplemented with 1% fetal bovine serum. Then, the medium was changed to fresh medium in the presence or absence of rhGDF-5, and cells were further incubated for 24 h. Supernatants were colleted from each well, and the quantity of VEGF was determined using a kit for mouse VEGF (mouse VEGF Immunoassay, TECHNE Corp., Minneapolis, MN, USA) according to the manufacturer's protocols. The minimal detectable dose of mouse VEGF was typically less than 3.0 pg/ml.

Statistical analysis

Data are expressed as means ± SD. Statistical significance of differences between groups was assessed by one-way analyses of variance (ANOVA).

Results

The effects of rhCI combined with rhGDF-5 14 days after injection

The rhCI appeared to allow the release of rhGDF-5 to form new bone in murine calvariae. In Fig. 1, new bone was formed at the rhGDF-5–rhCI composite-injected site, and it resulted in a bulge-like structure corresponding to the injected area (Fig. 1A). In Fig. 1(C), microcomputed tomography showed that new bone spread widely at the rhGDF-5–rhCI composite-injected site and was distinct from cortical bone, suggesting that new bone formed in response to the injection of rhGDF-5–rhCI composite. There was no bulge at the site injected with rhCI alone (Fig. 1B).

Histological observations

Serial sections prepared 3, 7 or 14 days after injection were observed histologically. Fourteen days after injection of rhGDF-5/rhCI composite, sections showed the newly formed bone-like structure on haematoxylin and eosin staining (Fig. 2B). There was no newly formed bone-like structure 3 or 7 days after injection of rhGDF-5–rhCI composite or rhCI alone (data not shown). In addition, injection of rhCI alone did not produce any newly formed bone-like tissues 14 days after injection (Fig. 2A). Alcian blue strongly stained the periosteum and the newly formed

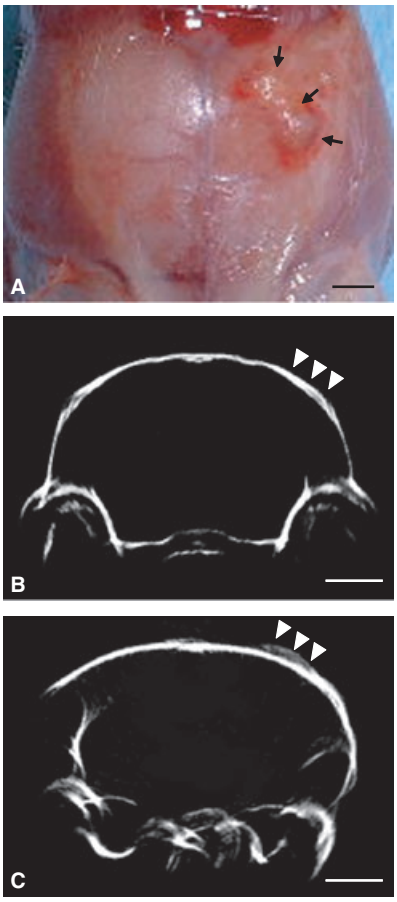

Fig. 1. Photographs of new bone formation in murine calvariae 14 days after the injection of rhGDF-5/rhCI composite or rhCI alone. (A) A bulge is confirmed at the injection site of the rhGDF-5/rhCI composite. The injected area is denoted by black arrows. (B) Two-dimensional microcomputed tomography did not show any newly formed bone at the site injected with rhCI alone (arrowheads). (C) Two-dimensional microcomputed tomography distinctly showed newly formed bone (arrowheads), different from existing bone, 14 days after the injection of rhGDF-5/rhCI composite. Bars: 2 mm.

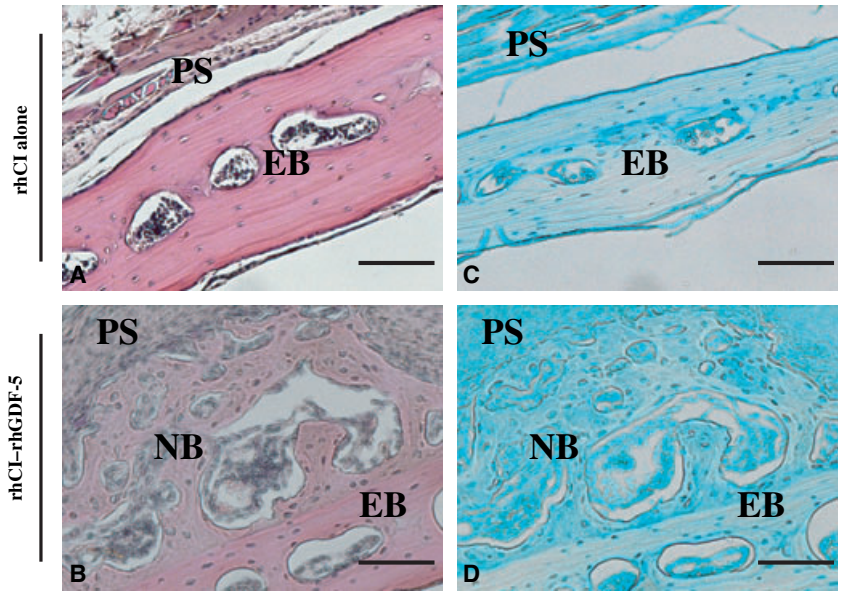

Fig. 2. Histological assessment of newly formed bone at the injection site of rhGDF-5–rhCI composite or rhCI alone, 14 days after injection. Serial sections were stained with haematoxylin and eosin (A,B) or alcian blue (C,D). Newly formed bone can be distinguished from existing bone at the injection site of rhGDF-5–rhCI composite. The periosteum and newly formed bone were strongly stained with alcian blue. No newly formed bone was observed 14 days after injection at the site injected with rhCI alone. Abbreviations: PS, periosteum; EB, existing bone; NB, newly formed bone. Scale bars represent 500 μm.

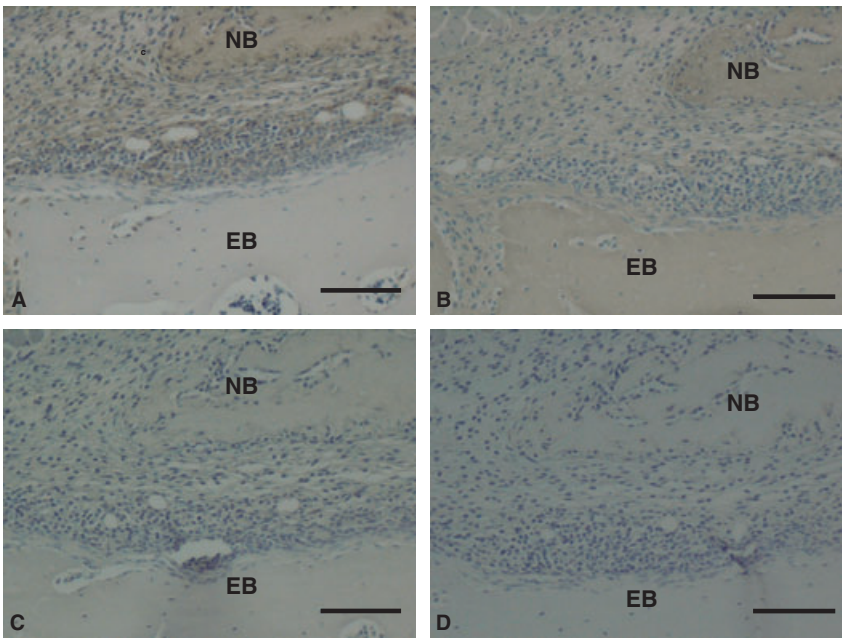

Fig. 3. Immunohistochemical staining for type I or type II collagen in newly formed bone-like tissues at the rhGDF-5–rhCI composite-injected site 14 days after injection. (A) Newly formed bone and peripheral tissues were positively stained with type II collagen antibody, whereas existing bone was not. (B) Existing bone (EB), newly formed bone (NB) and peripheral tissues were positively stained with type I collagen. Negative controls using isotype control IgG are shown in (C,D). Scale bars represent 200 μm.

bone-like tissues, indicating that they contained acid mucopolysaccharides (Fig. 2C,D).

Immunohistological observations 14 days after injection

Immunohistochemical staining for type I or II collagen, Sox9 and VWF were performed on sections from mice killed 14 days after injection. The staining pattern for collagen was quite different from the existing bone and newly formed bone-like tissues. Immunoreactivity for type I collagen was observed in both the existing bone and newly formed bone-like tissues, whereas type II collagen-positive staining was detected in the newly formed bone-like tissues and their peripheral area, indicating that endochondral ossification was induced by rhGDF-5 (Fig. 3). Sox9-positive cells were detected in the hypertrophic periosteum (Fig. 4). The periosteum at the rhCI-injected site was not hypertrophic and did not include any Sox9-positive cells (Fig. 4C). Sox9-positive cells appeared to be surrounded by newly formed bone-like tissues from the periosteum (Fig. 4B). Furthermore, immunohistochemical studies showed that a number of luminal structures made of VWF-positive cells were present in the tissues (Fig. 5).

Secretion of VEGF by primary osteoblasts, periosteal cells and connective tissue fibroblasts

The addition of rhGDF-5 significantly enhanced VEGF secretion in connective tissue fibroblasts, although it did not enhance secretion in osteoblasts and periosteal cells (Fig. 6). Fibroblast growth factor, as positive control, also induced an increase in VEGF in connective tissue fibroblasts (data not shown). These results suggested that connective tissue fibroblasts might play a central role in the secretion of angiogenic factors mediating the response to rhGDF-5.

Discussion

We showed that rhGDF-5 released from rhGDF-5–rhCI composite pro-

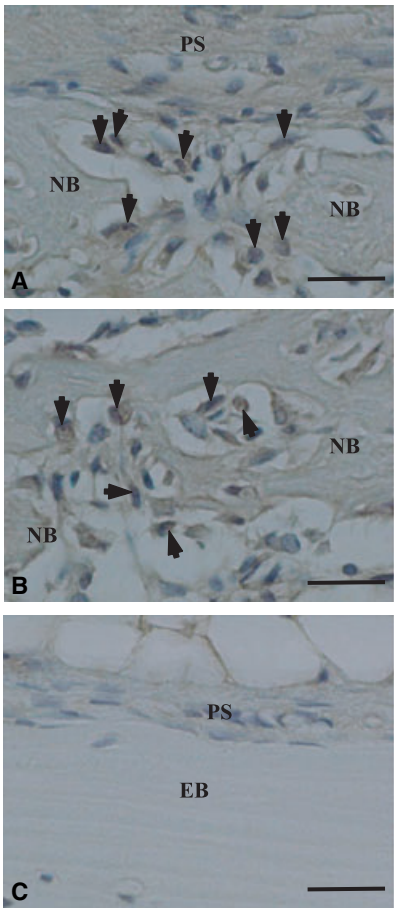

Fig. 4. Immunohistochemical analysis of expression of Sox9 14 days after injection of rhGDF-5–rhCI composite or rhCI alone. (A) Sox9-positive cells (arrows) are present in the hypertrophic periosteum and at the interface between the periosteum and newly formed bone-/cartilage-like tissues. (B) Sox9-positive cells (arrows) are also present in newly formed bone-/cartilage-like tissues. (C) No Sox9-positive cell was observed at the rhCI-injected site. Abbreviations: PS, periosteum; EB, existing bone; NB, newly formed bone. Scale bars represent 50 μm.

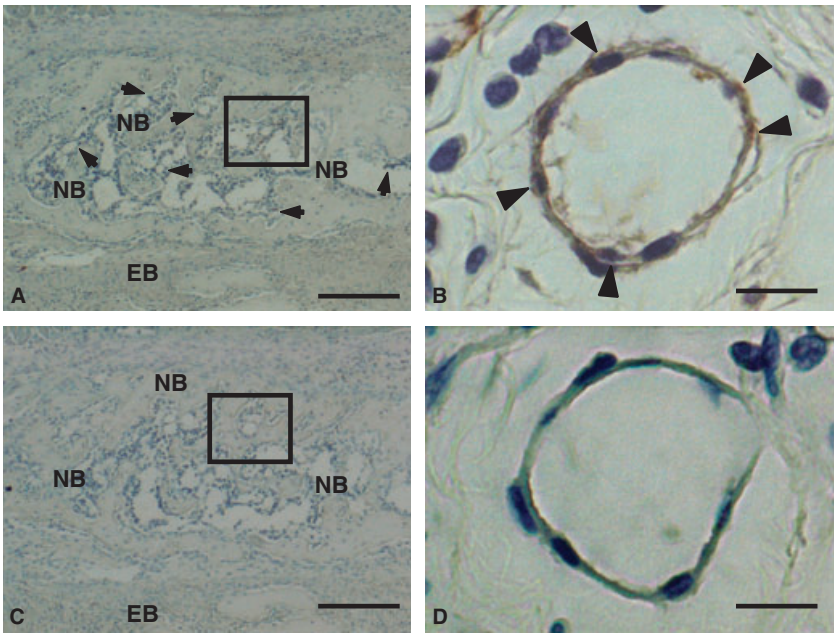

Fig. 5. Immunohistochemical staining for Von Willebrand Factor (VWF) in newly formed bone-/cartilage-like tissues. (A) A number of vessel luminae (arrows) were observed in newly formed bone-/cartilage-like tissues. (B) The luminae contained VWF-positive cells. (B) is a higher magnification of the box area in (A). (C,D) Negative control stained with isotype control IgG. (D) is a higher magnification of the box area in (C). Abbreviations: EB, existing bone; NB, newly formed bone. Scale bars represent 30 μm (A,C) and 50 μm (B,D).

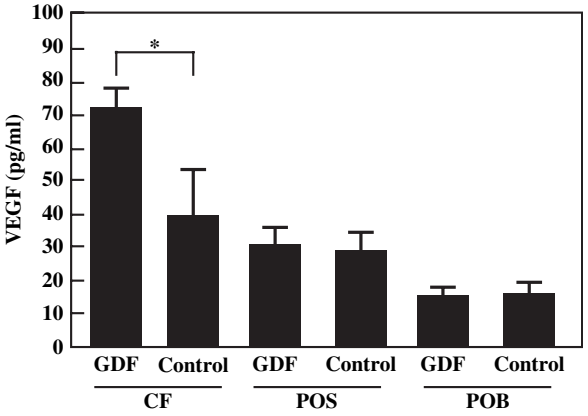

Fig. 6. Secretion of VEGF from primary osteoblasts, periosteal cells and connective tissue fibroblasts in the presence or absence of rhGDF-5. Increased VEGF secreted in skin fibroblasts in the presence of rhGDF-5 was evaluated in the supernatant after 48 h by enzyme-linked immunosorbent assay. $*P < 0.05$ (one-way ANOVA). Abbreviations: CF, primary connective tissue fibroblasts; POB, primary osteoblasts; POS, primary periosteal cells.

moted new bone formation via endochondral ossification in murine calvariae using a very simple injection technique. These results suggest that rhCI is helpful as a biomaterial for inducing bone formation in substitution for atelocollagen from animal tissues. Furthermore, we demonstrated that rhGDF-5 released from rhCI induced Sox9 expression and angiogenesis for endochondral ossification in murine calvariae.

Collagen plays an essential role in providing a scaffold for cellular support, thereby affecting cell attachment, migration, proliferation and differentiation. It is also often used in tissue engineering for bone or skin reconstruction. Currently, collagen used in tissue engineering applications is derived from animal tissues. It is atelocollagen that is produced by telopeptide removal from natural collagen molecules, resulting in extremely low antigenicity. However, the use of bovine collagen should be reconsidered and restricted because of the risks of bovine spongiform encephalopathy,

which is considered to be transmittable to humans. Accordingly, we estimated the effects of GDF-5 using recombinant human collagen, which can also be reconstituted into fibrils that can be formulated into three-dimensional gels.

In the present study, rhCI appeared to support the effects of rhGDF-5 atelocollagen we previously reported (25). At injection sites of rhGDF-5–rhCI composite, newly formed bone was induced within the initial 14 days, as seen radiologically (Fig. 1C). However, none of the new bone formation was induced at injection sites of rhCI alone (Fig. 1B). Newly formed bone-like tissues were strongly stained by alcian blue (Fig. 2D). Furthermore, these tissues showed weakly positive staining for type II collagen; however, the existing bone did not include type II collagen (Fig. 3). These results suggest that new bone was formed via endochondral ossification, as previously reported (25). Thus, rhCI may be helpful in providing the preferable effects for rhGDF-5 as a substitute of atelocollagen derived from animal tissues, indicating that the use of rhCI can avoid risks of viral and prion contamination and of unknown and potentially zoonotic infectious diseases.

Exogenous rhGDF-5 stimulates the proliferation of keratinocytes, fibroblasts, primary osteoblasts, periosteal cells and periodontal ligament cells *in vitro* (25–27). It has been reported that GDF-5 may be involved in the formation of periodontal ligament, especially the dental attachment apparatus (15,16). In a previous study, we showed that rhGDF-5 stimulated proliferation, alkaline phosphatase activity and sulphated glycosaminoglycan in human periodontal ligament cells, indicating that it may provide an environment favourable to periodontal healing or regeneration by affecting extracellular matrix metabolism (27). We also showed that rhGDF-5 induced endochondral ossification in murine calvariae using atelocollagen derived from porcine skin (25). Accordingly, new bone formation induced by rhGDF-5 in the present study encourages us to promote research of regenerative therapy using rhGDF-5 for periodontal disease. However, how endochondral ossification occurs in murine calvariae remains to by clarified. In the present *in vivo* study, a number of vessel luminae were observed in the newly formed bone, and the vessel luminae included VWF-positive cells (Fig. 5). We also assayed VEGF production using cultured calvaria-derived cells to examine whether GDF-5 is involved in the induction of angiogenesis. Angiogenic growth factor VEGF was enhanced in the presence of rhGDF-5. Among the calvaria-derived cells, connective tissue fibroblasts produced VEGF in response to rhGDF-5, suggesting that they may have contributed to angiogenesis in the present study (Fig. 6). These results indicate that injected rhGDF-5 promotes angiogenesis through the production of VEGF in murine calvariae. In another study, GDF-5 induced plasminogen activator activity and accelerated the migration of bovine aortic endothelial cells in a chemotactic fashion, which might contribute to the process of angiogenesis *in vivo* (27). It has been shown that GDF-5 promotes angiogenic activity in both chick chorioallantoic membranes and rabbit cornea assays (28). Angiogenesis plays a critical role in bone development and in postnatal fracture repair (29,30).

Differentiation and proliferation of osteogenic and chondrogenic mesenchymal cells are regulated by molecular signals (31). Growth/differentiation factor-5 increases the amounts of prechondrogenic mesenchymal cell condensation and cartilaginous nodules and causes a more sustained elevated expression level of Sox9 (32). Sox9 is a transcription factor belonging to the Sex-determining Region Y (SRY) family of high-mobility box proteins and plays a major role in endochondral ossification (33). In the present study, we demonstrated Sox9 expression in the hypertrophic periosteum and in the newly formed bone after injection of rhGDF-5–rhCI composite (Fig. 4A,B). These results suggest that subcutaneously injected rhGDF-5 stimulates mesenchymal cells in the periosteum and promotes Sox9 expression in endochondral ossification.

In conclusion, we have demonstrated that rhCI is suitable as a biomaterial to control GDF-5 release, which induces endochondral ossification via Sox9 expression in the periosteum and angiogenic activity in murine calvariae. The application of rhGDF-5–rhCI composite may be effective to induce bone-/cartilage-like tissues through endochondral ossification.

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References

1. Wissink MJ, Beernink R, Poot AA *et al.* Improved endothelialization of vascular grafts by local release of growth factor from heparinized collagen matrices. *J Control Release* 2000;**64:**103–114.
2. Zisch AH, Schenk U, Schense JC, Sakiyama-Elbert SE, Hubbell JA. Covalently conjugated VEGF–fibrin matrices for endothelialization. *J Control Release* 2001; **72:**101–113.
3. Fertala A, Sieron AL, Ganguly A *et al.* Synthesis of recombinant human procollagen II in a stably transfected tumour cell line (HT1080). *Biochem J* 1994;**298:**31–37.
4. Chen M, Costa FK, Lindvay CR, Han YP, Woodley DT. The recombinant expression of full-length type VII collagen and characterization of molecular mechanisms underlying dystrophic epidermolysis bullosa. *J Biol Chem* 2002;**277:**2118–2124.
5. Tomita M, Kitajima T, Yoshizato K. Formation of recombinant human procollagen I heterotrimers in a baculovirus expression system. *J Biochem (Tokyo)* 1997;**121:**1061–1069.
6. Nokelainen M, Helaakoski T, Myllyharju J *et al.* Expression and characterization of recombinant human type II collagens with low and high contents of hydroxylysine and its glycosylated forms. *Matrix Biol* 1998;**16:**329–338.
7. Buechter DD, Paolella DN, Leslie BS *et al.* Co-translational incorporation of trans-4-hydroxyproline into recombinant proteins in bacteria. *J Biol Chem* 2003; **278:**645–650.
8. Yang C, Hillas PJ, Baez JA *et al.* The application of recombinant human colla-

gen in tissue engineering. *Biodrugs* 2004;**18:**103–119.
9. 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.
10. 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.
11. 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.
12. 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.
13. Storm EE, Huynh TV, Copeland NG *et al*. Limb alterations in *brachypodism* mice due to mutations in a new member of the TGFβ-superfamily. *Nature* 1994;**368:**639–643.
14. Chang SC, Hoang B, Thomas JT *et al*. Cartilage-derived morphogenetic proteins. New members of the transforming growth factor-β superfamily predominantly expressed in long bones during human embryonic development. *J Biol Chem* 1994;**269:**28227–28234.
15. 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.
16. 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.
17. Buxton P, Edwards C, Archer CW, Francis-West P. Growth/differentiation factor-5 (GDF-5) and skeletal development. *J Bone Joint Surg Am* **83-A** Suppl. 1**:**S23–S30.
18. Archer CW, Dowthwaite GP, Francis-West P. Development of synovial joints. *Birth Defects Res C Embryo Today* 2003;**69:**144–155.
19. 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.
20. Edwards CJ, Francis-West PH. Bone morphogenetic proteins in the development and healing of synovial joints. *Semin Arthritis Rheum* 2001;**31:**33–42.
21. Coleman CM, Tuan RS. Functional role of growth/differentiation factor 5 in chondrogenesis of limb mesenchymal cells. *Mech Dev* 2003;**120:**823–836.
22. 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-β gene family. *J Clin Invest* 1997;**100:**321–330.
23. 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.
24. Kuniyasu H, Hirose Y, Ochi M *et al*. Bone augmentation using rhGDF-5-collagen composite. *Clin Oral Implants Res* 2003;**14:**490–499.
25. Yoshimoto T, Yamamoto M, Kadomatsu H *et al*. Recombinant human growth/differentiation factor-5 (rhGDF-5) induced bone formation in murine calvariae. *J Periodont Res* 2006;**41:**140–147.
26. Kim DS, Korting HC, Schafer-Korting M. Effects of growth factors on the proliferation of human keratinocytes and fibroblasts in vitro. *Pharmazie* 1998;**53:** 51–57.
27. Nakamura T, Yamamoto M, Tamura M, Izumi Y. Effects of growth/differentiation factor-5 on human periodontal ligament cells. *J Periodont Res* 2003;**38:** 597–605.
28. Yamashita H, Shimizu A, Kato M *et al*. Growth/differentiation factor-5 induces angiogenesis in vivo. *Exp Cell Res* 1997;**235:**218–226.
29. Colnot CI, Helms JA. A molecular analysis of matrix remodeling and angiogenesis during long bone development. *Mech Dev* 2001;**100:**245–250.
30. Zelzer E, McLean W, Ng YS *et al*. Skeletal defects in VEGF (120/120) mice reveal multiple roles for VEGF in skeletogenesis. *Development* 2002;**129:** 1893–1904.
31. Olsen BR, Reginato AM, Wang W. Bone development. *Annu Rev Cell Dev Biol* 2000;**16:**191–220.
32. Hatakeyama Y, Tuan RS, Shum L. Distinct functions of BMP4 and GDF5 in the regulation of chondrogenesis. *J Cell Biochem* 2004;**91:**1204–1217.
33. Wright E, Hargrave MR, Christiansen J *et al*. The *Sry*-related gene *Sox9* is expressed during chondrogenesis in mouse embryos. *Nat Genet*, 1995;**9:**15–20.

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