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Platelet-derived growth factor and bone morphogenetic protein in the healing of mandibular fractures in rats

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SUMMARY. **We studied the effects of platelet-derived growth factor-B (PDGF-B) and bone morphogenetic protein-2 (BMP-2) during the healing of mandibular closed fractures in rats by immunohistochemical methods. Unilateral closed fractures were created in the mandibles of thirty 12-week-old rats. BMP-2 was expressed during all stages of healing, but PDGF-B was expressed mainly in the early and middle stages, and not in the later stage of the healing process. We conclude that PDGF-B was associated with the proliferation and migration of primitive mesenchymal cells. BMP-2 was related to the differentiation of mesenchymal cells into osteoblasts and chondroblasts. PDGF-B and BMP-2 both have distinct regulatory effects on the healing of fractures.**

Keywords: **PDGF; BMP-2; Mandibular fracture healing**

INTRODUCTION

The healing of a fracture is a sequential process to restore normal structure and function or is regulated by both local and systemic factors. To promote healing, a number of growth factors and cytokines are localised in and around the fracture site. It has been suggested that specific growth factors and cytokines may regulate fracture healing during the different stages.[1] The histological features of healing bone can be divided into the initial haematoma, the formation of callus and remodelling of bone. Callus comprises intramembranous and endochondral ossification. Intramembranous ossification is the formation of new bone in the periosteum, which occurs by differentiation of osteoblasts from osteoprogenitor cells without an intermediate cartilaginous stage. Endochondral ossification involves the development of cartilage, which is later calcified and replaced by bone. Remodelling is the result of the actions of osteoclasts and osteoblasts and results in the restoration of form and function of the mature bone.[2,3]

Platelet-derived growth factor (PDGF) is a homodimeric or heterodimeric protein with A and B polypeptide chains that has three possible isoforms: PDGF-AA, PDGF-BB and PDGF-AB.[4–6] PDGF is a heat-stable positively charged protein produced by osteoblasts, platelets and monocytes/macrophages. PDGF-B has higher mitogenic and chemotactic potential and also a higher affinity to bone matrix than PDGF-A.[1,4–7] This is in part because of the ability of PDGF-B to bind to both α- and β-receptors of PDGF, whereas PDGF-A occupies only the α-receptor.[8,9] PDGF-B has a potent effect on migration and proliferation of cells, and can promote periodontal regeneration.[10,11]

Bone morphogenetic protein (BMP) is a subfamily of the transforming growth factor-β (TGF-β) superfamily.[12–14] BMPs are promising osteoinductive substances and are expected to be used clinically for reconstruction of bone. BMPs are expressed in several non-skeletal organs and tissues, such as tooth buds, the central nervous system, and in the oral and maxillofacial area of the human embryo.[15–17] The efficacy of BMPs in inducing bone formation *in vivo* at both bony and non-bony sites has been widely studied. BMP-2 is the most active and is able to induce the formation of new bone by affecting both the differentiating and proliferative functions of osteoblasts and chondrocytes.[18,19]

Although it is established that osteogenic precursor cells respond to cytokines and growth factors, their role in the healing of fractures has not been fully defined. Many studies of the process of fracture healing have been made by investigators with orthopaedic backgrounds, but most of these have been on long bones, such as the tibia and femur. In our field, mandibular fractures are common and we usually use metal plates to fix the fractures. To predict surgical results, a knowledge of the normal process of fracture healing is essential. We have studied the healing

process in closed mandibular fractures in rats and investigated the types of cells that produce PDGF-B and BMP-2.

MATERIAL AND METHODS

Animals

Thirty male Wistar rats aged 12 weeks (SLC Co, Ltd, Fukuoka, Japan) and weighing 320–350 g were used in this study. The rats were maintained in temperature-controlled rooms and given unrestricted access to food and water. They were caged in pairs. The experimental procedures followed the guiding principles for the care and use of animals described in the Kyushu University Journal of Animal Care.

Procedure and preparation of tissue

Under intraperitoneal anaesthesia using pentobarbitone (40 mg/kg) (Nembutal, Abbot Laboratories Co, Ltd, Chicago, IL, USA), the right side of the mandibular ramus was fractured with a bending clamp. On day 3 ($n = 8$), day 7 ($n = 8$), day 14 ($n = 8$) and day 21 ($n = 6$) after operation the animals were killed by deep ether anaesthesia. After perfusion, each mandible was excised, fixed in 4% paraformaldehyde overnight at 4 °C, decalcified with 20% EDTA, pH 7.4, at 4 °C and embedded in optimum cutting temperature compound (Sakura Finetechnical Co, Ltd, Tokyo, Japan) to yield a frozen specimen. The specimens were kept at −80 °C until cryosectioning. The specimens were cut frontally (5 μm thick) with a Cryostat, placed on slide glasses coated with 3-aminopropyl-triethoxy-silane (Sigma Chemical Co, Ltd, Steinheim, Germany), and dried overnight at room temperature. Representative sections for each experiment were stained with haematoxylin and eosin.

Antibodies

Affinity-purified goat polyclonal anti-BMP-2 antibody and rabbit polyclonal anti-PDGF-B antibody were bought from Santa Cruz Biotech (Santa Cruz, CA, USA). Anti-PDGF-B antibody is raised against a recombinant protein corresponding to amino acids 136–139 mapping at the carboxy terminus of mature PDGF-B of human origin.

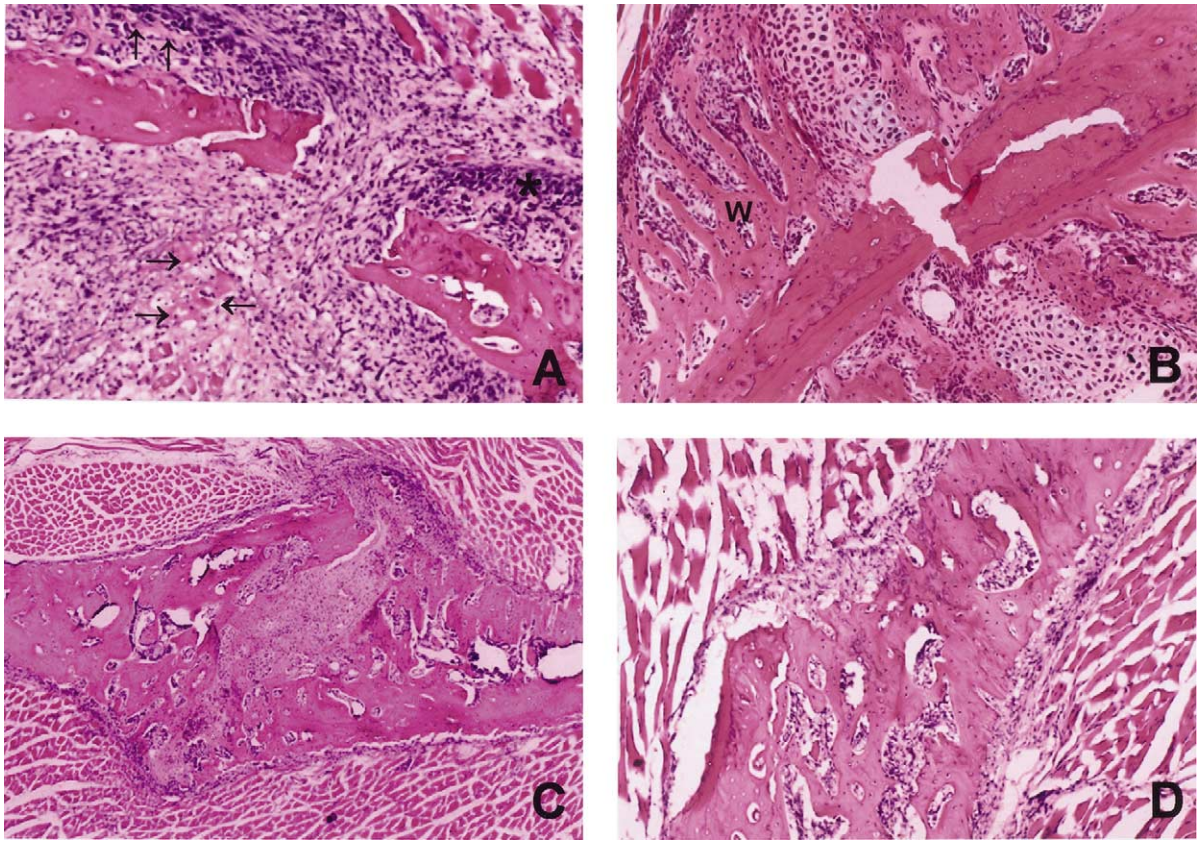

Fig. 1 On day 3 after the fracture, condensed mesenchymal cells in the periosteum (asterisk) and the haematoma (horizontal arrows) were seen around the fracture site. There was new woven bone in the periosteum (vertical arrows) (A). On day 7 after the fracture, woven bone (W) had increased and the fracture gap was bridged by cartilage (B). On day 14 after the fracture, trabecular bone had advanced towards and spanned the fracture. Cartilaginous callus was developing (C). On day 21 after the fracture, woven bone was replaced with lamellar bone (D). Haematoxylin and eosin staining; A, B, D: original magnification × 40; C: original magnification × 25.

Immunohistochemistry

Immunohistochemistry was by the avidin–biotin peroxidase complex method. After the sections had been rehydrated, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in absolute methanol for 20 minutes at room temperature. After washing with phosphate-buffered saline, sections were incubated with 3% donkey serum for 30 minutes at room temperature to eliminate non-specific protein binding, and then with the primary antibody at 4 °C overnight in a humidified chamber. After they had been washed with phosphate-buffered saline, the sections were incubated with a biotinylated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) for 1 hour and an avidin–biotin peroxidase complex (DAKO Japan, Kyoto, Japan) for 30 minutes. Finally, the sections were developed with 3,3′-diaminobenzidine (DAB substrate kit, Vector Lab., Burlingame, CA, USA) and confirmed under a microscope. Counter staining was by haematoxylin. As negative controls we used normal goat IgG, normal rabbit IgG and normal mouse IgG instead of the primary antibodies.

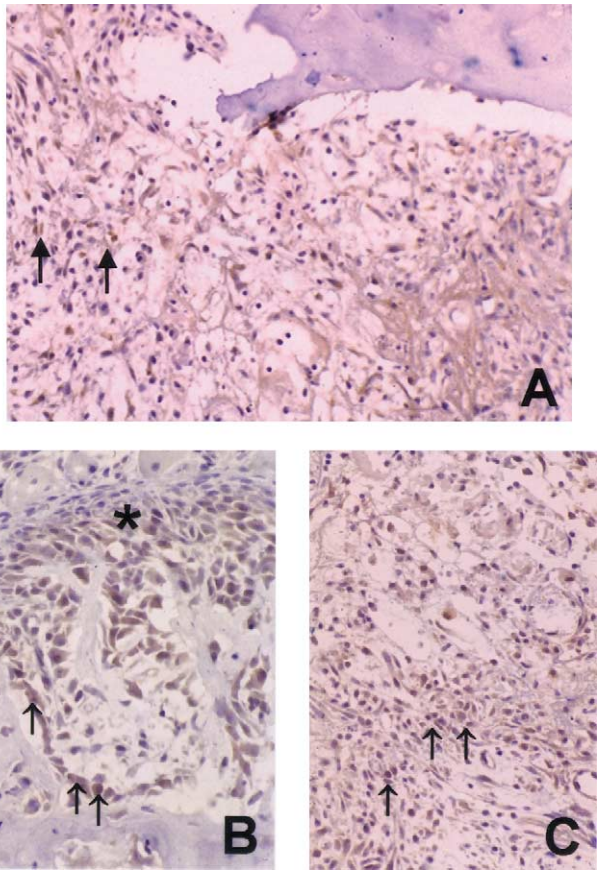

Fig. 2 Immunolocalisation of PDGF-B (A) and BMP-2 (B) and (C) on day 3 after the fracture. PDGF-B was seen in some macrophage-like cells (arrows) (A). Osteoblasts lining woven bone stained for BMP-2 (arrows) (B). Some mesenchymal progenitor cells near the haematoma were stained by BMP-2 (arrows) (C). Asterisk = periosteum. A, C: original magnification × 100; B: original magnification × 200.

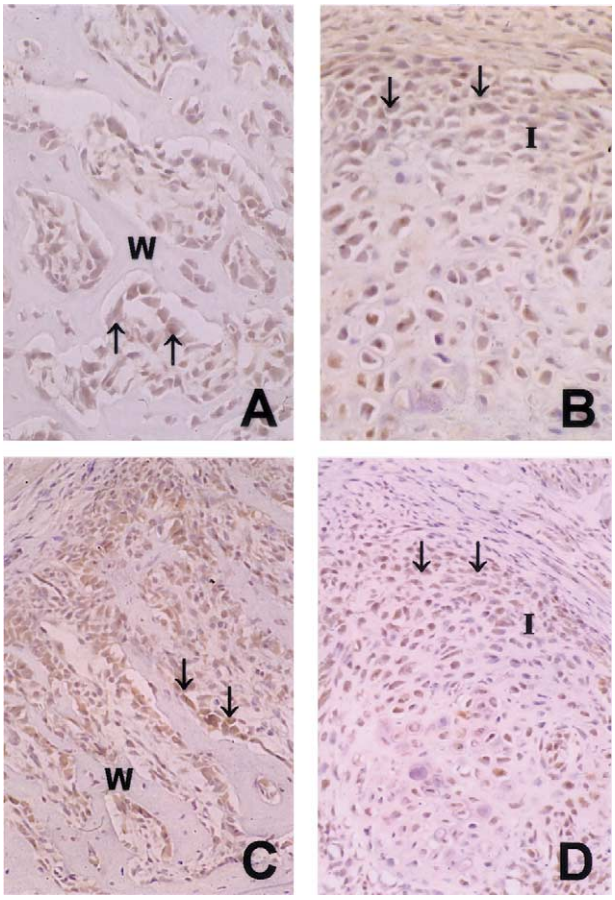

Fig. 3 Immunolocalisation of PDGF-B (A) and (B) and BMP-2 (C) and (D) on day 7 after the fracture. Osteoblasts (arrows) (A) and (C) lining woven bone stained strongly for PDGF-B and BMP-2 as did chondrocytes precursor cells (arrows) (B) and (D). I: immature chondrocytes, W: woven bone. A, B: original magnification × 200; C, D: original magnification × 100.

RESULTS

Histological findings

On day 3 after the fracture, a haematoma, including various inflammatory cells, was seen at the fracture site and a lot of primitive mesenchymal cells had gathered in and around the haematoma. The periosteum was thickened by the proliferation of periosteal cells proximal and distal to the fracture site (Fig. 1A). In the periosteum near the fracture site there was also an evidence of newly formed woven bone.

On day 7 after the fracture, there was an increase in newly formed woven bone in the intramembranous ossification area and there was a corresponding increase in the number of osteoblasts lining the newly formed woven bone. At the same time, the fracture gap was bridged by the endochondral ossification that proceeded from the initial fracture haematoma through formation of cartilage (Fig. 1B).

On day 14 after the fracture, the newly formed woven bone had become lamellar bone and there were fewer periosteal cells. The extracellular matrix surrounding the hypertrophic chondrocytes was calcified, and osteoblasts had begun to lay down new bone (Fig. 1C).

On day 21 after the fracture, the woven bone was replaced with lamellar bone, but the remodelling still continued (Fig. 1D).

Immunohistochemical findings

By day 3 after the fracture, there was staining for PDGF-B in some of the macrophage-like cells in the haematoma adjacent to the fracture site, but this was not present in the periosteal cells (Fig. 2A). On the other hand, most of the proliferating periosteal cells, except for the cells around the periosteum, showed staining for BMP-2 in the cytoplasm (Fig. 2B). Immunostaining for BMP-2 around the haematoma was minimal. Some primitive mesenchymal cells in the fracture gap were stained (Fig. 2C).

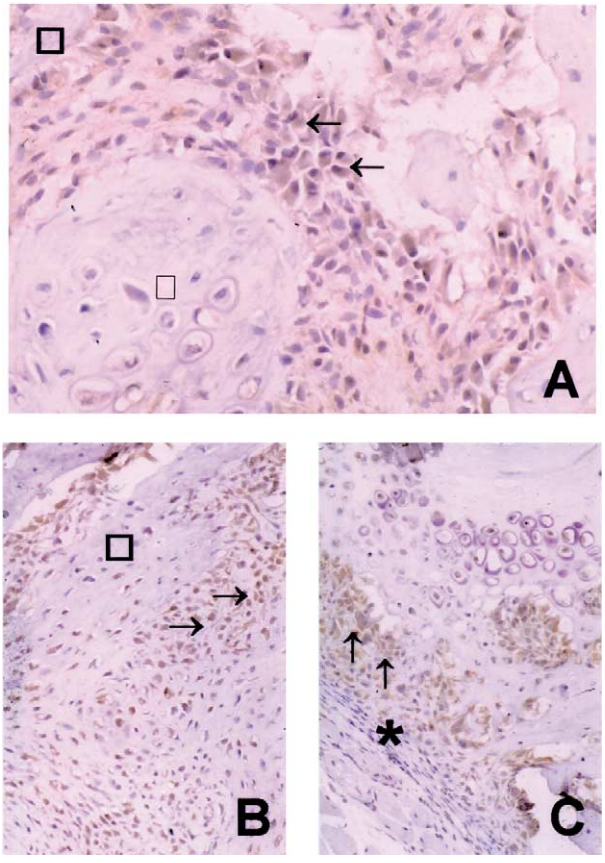

Fig. 4 Immunolocalisation of PDGF-B (A) and BMP-2 (B) and (C) on day 14 after the fracture. Osteoblasts lining the calcified cartilage matrix and woven bone were stained for PDGF-B (A: original magnification × 200) and for BMP-2 (B, C: original magnification × 100). Asterisk = periosteum, square = endochondral ossification area.

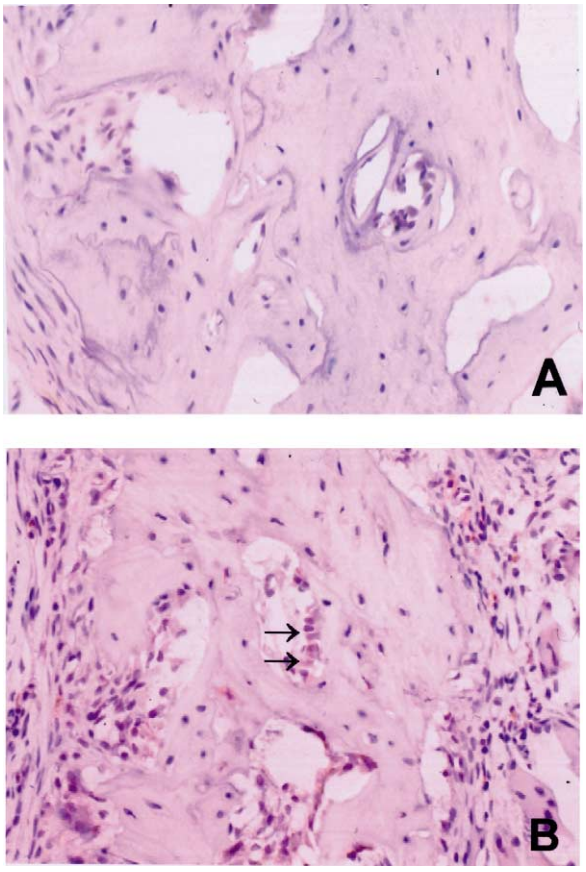

Fig. 5 Immunolocalisation of PDGF-B (A) and BMP-2 (B) on day 21 after the fracture. PDGF-B was not detected (A). Some osteoblasts (arrows) were stained by BMP-2 (B); original magnifications × 200.

By day 7 after the fracture, PDGF-B-stained and BMP-2-stained cells were common among the cells lining the newly formed woven bone (Fig. 3A and C). In the endochondral ossification area, precursor cells of chondrocytes showed the most intense staining for PDGF-B and BMP-2 (Fig. 3B and D).

By day 14 after the fracture, there was intense staining of PDGF-B and BMP-2 in the osteoblasts lining the calcified cartilage matrix and woven bone (Fig. 4A–C). The number of chondrocytes had increased, but the proportion that stained for PDGF-B and BMP-2 had decreased.

By day 21 after the fracture, PDGF-B was no longer detected (Fig. 5A). The osteoblasts lining the trabecular bone were still stained for BMP-2 (Fig. 5B).

DISCUSSION

Although it is established that bone cells produce and respond to PDGF-B and BMP-2, the role of these factors in the healing of fractures has not been fully defined. In our study, PDGF-B staining was detected in macrophages

and in some primitive mesenchymal cells around the haematoma on day 3 after the fracture. These findings support the hypothesis that PDGF-B is a prototype wound-healing cytokine because it is stored in circulating platelets and released during platelet degranulation at the site of vascular injury and acts as a chemotactic agent.[9,20] In the middle stage of fracture healing, staining for PDGF-B was noted in osteoblasts lining the newly formed woven bone and in chondrocytes in the cartilaginous callus. We therefore suggest that PDGF-B has a strong effect on the proliferation of osteoblasts and chondrocytes, and also has an important role in stimulating chemotaxis of cells.

BMP-2 has effects that are different from those of PDGF-B. In the initial stages of fracture healing, BMP-2 staining was detected mainly in the active cuboidal cells that were lining the newly formed woven bone and also in some primitive mesenchymal cells around the fracture haematoma. As newly formed woven bone is formed in the periosteum from the early stage of fracture healing and proceeds at the site of fracture, we suggest that BMP-2 stimulates the primitive mesenchymal cells to differentiate to osteoblasts and chondrocytes for formation of callus. On day 7 after the fracture, BMP-2 and PDGF-B staining were detected in osteoblasts that were lining newly formed woven bone in the intramembranous ossification area and immature chondrocytes in endochondral ossification area. This must be the most active stage of fracture healing. The localisation of these cytokines suggests that there is an intimate interplay between PDGF-B and BMP-2 during this stage, in which they both act as autocrine and paracrine agents.

We showed that PDGF-B participated in the proliferation of osteoblasts and chondrocytes. On the other hand, we found that BMP-2 participated in all stages of fracture healing and seemed to play an important part in the formation of intramembranous ossification as well as in the differentiation of chondrocytes and osteoblasts. As the mature lamellar bone replaced the woven bone, in both intramembranous ossification and endochondral ossification, the staining of PDGF-B and BMP-2 also decreased in the later stage. Bostrom[18] and Spector *et al*.[21] confirmed a similar pattern of the BMP-2 immunolocalisation, with intense staining in the first week after the fracture, which decreased soon after lamellar bone had replaced the woven bone.

The most important difference between PDGF and BMPs is that PDGF isoforms, unlike BMPs, are ineffective in initiating the formation of bone by themselves.[22,23] Previous studies have reported that PDGF increased the volume of bony tissues *in vivo*, although *in vitro* studies showed suppression of osteoblastic differentiation as well as stimulation of cell proliferation.[24,25] In healing fractures in the rat mandible, PDGF-B was strongly expressed in osteoblasts that lined the newly formed woven bone and also chondrocyte precursors, suggesting stimulatory effects of PDGF-B on the formation of bone. Taken together, there is no doubt that BMP-2 and PDGF-B have a pivotal role during the proliferation and differentiation of osteoblasts and chondrocytes during the healing of mandibular fractures.

In conclusion, PDGF-B and BMP-2 have different biological functions during the healing of mandibular fractures. However, further study is needed to characterise the interactions of these factors with each receptor; the results of such a study will aid our understanding of the functional roles of molecular regulation in various morphogenetic processes during the formation of bone. Thorough understanding of the mechanism and the molecular regulation during the healing of mandibular fractures will help surgeons to expedite the healing process.

REFERENCES

1. Hollinger J, Wong Mark KE. The integrated processes of hard tissue regeneration with special emphasis on fracture healing. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1996; 82: 594–606.
2. Einhorn TA. The cell and molecular biology of fracture healing. Clin Orthop 1998; 355 (Suppl): S7–S21.
3. Volk SW, Leboy PS. Regulating the regulators of chondrocytes hypertrophy. J Bone Miner Res 1999; 14: 483–486.
4. Fujii H, Kitazawa R, Maeda S, Mizuno K, Kitazawa S. Expression of platelet derived growth factor proteins and their receptor α and β mRNAs during fracture healing in the normal mouse. Histochem Cell Biol 1999; 112: 131–138.
5. Claesson-Welsh L, Heldin CH. Platelets derived growth factor. Three isoforms that bind to two distinct cell surface receptors. Acta Oncol 1989; 28: 331–334.
6. Centrella M, McCarthy TL, Kusmik WF, Canalis E. Isoform-specific regulation of platelet derived growth factor activity and binding in osteoblasts-enriched culture from fetal rat bone. J Clin Invest 1992; 89: 1076–1084.
7. Barnes GL, Kostenuik PJ, Gerstenfeld LC, Einhorn TA. Growth factor regulation of fracture repairs. J Bone Miner Res 1999; 14: 1805–1815.
8. Zhang L, Leeman E, Carnes DC, Graves DT. Human osteoblasts synthesize and respond to platelet-derived growth factor. Am J Physiol 1991; 261: C348–C354.
9. Canalis E, McCarthy T, Centrella M. Growth factors and the regulation of bone remodeling. J Clin Invest 1988; 81: 277–281.
10. Lynch SE, Williams RC, Polson AM *et al*. A combination of platelet-derived and insulin-like growth factors enhances periodontal regeneration. J Clin Periodontol 1989; 16: 545–548.
11. Rutherford RB, Niekrash CE, Kennedy JE, Charette MF. Platelet-derived and insulin-like growth factors stimulate regeneration of periodontal attachment in monkeys. J Periodontal Res 1992; 27: 285–290.
12. Rosier RN, O'Keefe RJ, Hicks DG. The potential role of transforming growth factor β in fracture healing. Clin Orthop 1998; 355 (Suppl): S294–S300.
13. Joyce ME, Jingushi S, Bolander ME. Transforming growth factor-β in the regulation of fracture repair. Orthop Clin North Am 1990; 21: 199–209.
14. Sakou T. Bone morphogenetic proteins: from basic studies to clinical approaches. Bone 1998; 22: 591–603.
15. Jones CM, Lyons KM, Hogan BLM. Involvement of bone morphogenetic protein-4 (BMP-4) and Vgr-1 in morphogenesis and neurogenesis in the mouse. Development 1991; 111: 531–542.
16. Thomadakis G, Ramoshebi LN, Crooks J, Rueger DC, Ripamonti U. Immunolocalization of bone mophogenic protein-2 and -3 and osteogenic protein-1 during murine tooth root morphogenesis and in other craniofacial structures. Eur J Oral Sci 1999; 107: 368–377.

17. Suzuki T, Bessho K, Segami N, Iizuka T, Nojima T. Immunohistochemical localisation of bone morphogenetic-2 in the oral and maxillofacial area of the human embryo. Br J Oral Maxillofac Surg 2001; 39: 289–293.
18. Bostrom MPG. Expressions of bone morphogenetic proteins in fracture healing. Clin Orthop 1998; 355 (Suppl): S116–S123.
19. Onishi T, Ishidou Y, Nagamine T *et al*. Distinct and overlapping patterns of localization of bone morphogenetic protein (BMP) family members and a bmp type II receptor during fracture healing in rats. Bone 1998; 22: 605–612.
20. Siegbahn A, Hammacher A, Westermark B, Heldin CH. Differential effects of the various isoforms of platelet-derived growth factor on chemotaxis of fibroblasts, monocytes, and granulocytes. J Clin Invest 1990; 85: 916–920.
21. Spector JA, Luchs JS, Mehrara BJ *et al*. Expression of bone morphogenetic proteins during membranous bone healing. Plast Reconstr Surg 2000; 107: 124–134.
22. Urist MR. Bone: formation by autoinduction. Science 1965; 150: 893–899.
23. Si XH, Jin Y, Yang LJ, Tipoe GL, White FH. Expression of BMP-2 and GF-β1 mRNA during healing of the rabbit mandible. Eur J Oral Sci 1997; 105: 325–330.
24. Bolander ME. Regulation of fracture repair by growth factor. Proc Soc Exp Biol Med 1992; 200: 165.
25. Yu X, Hsieh SC, Bao W, Graves DT. Temporal expression of PDGF receptors and PDGF regulatory effects on osteoblastic cells in mineralizing cultures. Am J Physiol 1997; 272: C1709–C1716.

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