IOP PUBLISHING BIOMEDICAL MATERIALS

Biomed. Mater. **3** (2008) 015008 (10pp) doi:10.1088/1748-6041/3/1/015008

Effect of BMP-2 and BMP-7 homodimers and a mixture of BMP-2/BMP-7 homodimers on osteoblast adhesion and growth following culture on a collagen scaffold

Claude Laflamme and Mahmoud Rouabhia

Groupe de recherche en écologie buccale, Faculté de médecine dentaire, Université Laval, Québec City, Québec G1K 7P4, Canada

E-mail: Mahmoud.rouabhia@fmd.ulaval.ca

Received 8 November 2007
Accepted for publication 7 January 2008
Published 6 February 2008
Online at stacks.iop.org/BMM/3/015008

Abstract

In the present study, we studied the involvement of BMP-2 and BMP-7 as homodimers and as a mixture of homodimers in bone regeneration using an engineered bone model. The engineered bone model consisted of a collagen scaffold populated with osteoblasts that acted as a carrier for the BMPs. BMP-2, BMP-7 and a mixture of BMP-2/BMP-7 were used at final concentrations of 10 and 100 ng ml^{-1}. Osteoblasts seeded onto a collagen scaffold were cultured for 24 h before being stimulated with the BMPs. Four days later, osteoblast adhesion to and growth on the scaffold were assessed. Osteocalcin, IL-6, metalloproteinase (MMP-2 and MMP-9) and protease inhibitor (TIMP-1 and TIMP-2) mRNA and protein levels were measured. Our results showed that the BMP-2, BMP-7 and a mixture of BMP-2/BMP-7 all promoted osteoblast growth on the collagen scaffold, with the mixture of BMP-2/BMP-7 enhancing the most growth. BMP-2 and the mixture of BMP-2/BMP-7 enhanced osteocalcin (an osteoblast differentiation marker) mRNA expression and protein secretion, likely via the IL-6 pathway given that IL-6 secretion was upregulated by BMP-7 and a mixture of BMP-2/BMP-7. BMPs promote extracellular matrix production by inhibiting MMP-2 mRNA and increasing TIMP-1 and TIMP-2 mRNA expressions and protein secretions. BMP-2, BMP-7 and the mixture of BMP-2/BMP-7 could promote bone regeneration via different mechanisms involving IL-6 and MMP inhibitors.

Introduction

Bone is able to regenerate spontaneously if the defect does not exceed a certain degree of damage. Bone defects can result from congenital deformities (cleft palate, facial clefts and facial asymmetry), trauma, tumour resection and degenerative diseases [1, 2]. Bone regeneration is often required to restore and improve the quality of life of patients. Over half a million patients/year in the United States require surgery to repair bone defects [3]. Non-repair of bone defects can result in devastating consequences ranging from disfigurement to loss of function and loss of limb [4]. Bone may not regenerate spontaneously following large defects. In such cases, surgery is required to repair the defect using bone grafts or bone graft substitutes in order to restore bone form and function [5]. One approach to restoring bone form and function is substituting bony material with permanent metal, ceramic, polymer or composite orthopaedic implants. These materials can be effective but have some limitations in terms of function, osteointegration and interactions with the host [2]. However, much work remains to be done to optimize bone form and function restoration and osteointegration techniques.

The restoration of body function by bone regeneration is a fundamental regenerative strategy to achieve full physical and physiological bone function.

The gold standard in bone defect management is autografting, which involves transferring of bone from one location to another in the same person. Autografting promotes significant bone regeneration [6] but involves considerable risks, including invasive pain and morbidity, infection and longer operating times with higher costs. Moreover, autografting is ineffective when the damaged bone volume exceeds the volume of the donor site [7]. The common alternatives to autografting are allografts (human cadaver bone) and xenografts (animal bone). However, these implants have additional limitations, including tissue immunogenicity, excessive resorption and potential disease transmission [8]. There is thus a growing need to find effective alternatives for bone defect management. *In vitro* engineered bone may be one such alternative for restoring bone defects. Engineering bone using osteoblasts can be done using natural or artificial scaffolds and various growth factors such as bone morphogenetic proteins (BMPs) [2]. BMPs are secreted proteins that are members of the transforming growth factor beta group. BMPs are a family of pleiotropic signalling molecules that play critical roles at various stages in the formation of a variety of tissues and organs, including bone. Different BMPs have been identified [9]. They are grouped into three subfamilies (BMP-2 and BMP-4), (BMP-5 to BMP-8) and (BMP-3 and GDF-10) based on amino acid sequence similarities [10]. Intramuscular and subcutaneous implantation studies looking at the osteoinductive properties of BMPs have revealed that they are involved in several events during bone morphogenesis (bone remodelling, bone formation, chondrogenesis and mesenchymal cell infiltration and proliferation) [11, 12]. BMPs are active both as homodimers and heterodimers [13, 14]. Native BMPs are expressed by many cells in bone fracture sites undergoing healing [15, 16], suggesting that they have local regulatory roles during bone repair. It has been reported that some BMPs have mitogenic activity in the presence of periosteal cells and chondrocytes [17]. For example, BMP-2 and BMP-4 induce significant cell proliferation, while BMP-5 and BMP-6 do not. The most potent osteogenic BMPs are BMP-2, BMP-4, BMP-6 and BMP-9 [18]. It has been reported that rhBMP-2 induces many of the events necessary for both intramembranous and endochondral ossification. The effects of rhBMP-2 on bone formation are more potent than those of rhBMP-4 or rhBMP-7 [19]. Interestingly, rhBMP-2 upregulates genes associated with osteogenic differentiation and downregulates genes associated with myogenic differentiation [20] as early as the first 24 h following stimulation [21]. Various studies have revealed that BMP heterodimers are much more osteogenic than homodimers. Different reports suggest that heterodimeric BMP proteins stimulate greater *in vitro* and *in vivo* activity than corresponding homodimers [13, 22–24]. Cells secreting heterodimers such as BMP-2/6, BMP-2/7 or BMP-4/7 have higher alkaline phosphatase activity as compared with other heterodimeric combinations or corresponding homodimers [13, 22]. Furthermore, BMP-2/7 heterodimers induce maximal alkaline phosphatase activity when BMP-2 and BMP-7 are infected at a 1:1 ratio [13]. *In vivo*, BMP-2/6, BMP-2/7 and BMP-4/7 heterodimers promote more significant ectopic bone formation than any of the corresponding homodimers. The highest effect was obtained with BMP-2/7 heterodimers [13].

Recombinant forms of human BMP-2 and BMP-7 have been produced for clinical use [25, 26]. BMPs can exert their biological effect and provide an initial substratum for the growth and differentiation of bone-forming cells when combined with an efficient delivery system [12]. Suitable carrier materials are essential for controlling bone mass formation [27]. BMP carriers have to be biocompatible, non-toxic, non-immunogenic and non-carcinogenic. For example, rhBMP-2 combined with a collagen sponge matrix carrier has proven to be effective for therapeutic applications [28]. Available studies related to the BMP effects on osteoblasts have been generated on monolayer culture which is rather far from reality. To mimic physiological conditions for bone regeneration, we introduced an extracellular matrix as a scaffold for osteoblast culture and as a carrier for BMPs. Using this model, we investigated the effect BMP-2, BMP-7 and a mixture of BMP-2/BMP-7 homodimers on osteoblast adhesion and proliferation and different mediator expression and production.

Experimental procedures

MG-63 osteoblast cell cultures

The MG-63 human osteosarcoma cell line is widely used to study the biocompatibility of orthopaedic and dental biomaterials. MG-63 osteoblast-like cells (American Type Culture Collection, Manassas, VA, USA) were subcultured in a 3:1 mixture of Dulbecco-Vogt's modified Eagle's (DME) medium and Ham's F-12 (H) (Invitrogen Life Technologies, Burlington, ON, Canada) supplemented with 24.3 μg ml^{-1} adenine, 10 μg ml^{-1} human epidermal growth factor (Chiron Corp., Emeryville, CA, USA), 0.4 μg ml^{-1} hydrocortisone (Calbiochem, La Jolla, CA, USA), 5 μg ml^{-1} bovine insulin, 5 μg ml^{-1} human transferrin, 2 $\times$ 10^{-9} M 3,3′,5′-triiodo-l-thyronine, 10^{-10} M cholera toxin (Schwarz/Mann, Cleveland, OH, USA), 100 U ml^{-1} penicillin, 25 μg ml^{-1} gentamicin (Schering, Pointe-Claire, QC, Canada) and 10% foetal calf serum (NCS, fetal clone II; Hyclone, Logan, UT, USA). Subconfluent cell cultures were trypsinized and split 1:10 to maintain cell growth and avoid differentiation. The cell cultures were incubated in a humid 5% CO_2 atmosphere at 37 °C.

Collagen membrane scaffolds

CollaTape® scaffolds (Zimmer Dental Corp., Mississauga, ON, Canada) were used. CollaTape® is fabricated using collagen from bovine deep flexor (Achilles) tendon. It is a sterile, pyrogen-free, biodegradable biomaterial available in 10 $\times$ 2.5 $\times$ 7.5 cm strips suitable for clinical use. It is a porous material that becomes gel-like when exposed to fluids. More than 90% of the CollaTape® consists of open pores, which can

Table 1. Primer sequences used for the RT-PCR.

Primers	Direction	Sequences	Length (bp)	References
IL-1β	Forward	5′-AGTACCTGAGCTCGCCAGTG-3′	802	[30]
	Reverse	5′-CTGCTTGAGAGGTGCTGATG-3′		
IL-6	Forward	5′-TCAATGAGGAGACTTGCCTG-3′	260	[31]
	Reverse	5′-GATGAGTTGTCATGTCCTGC-3′		
MMP-2	Forward	5′-GTGCTGAAGGACACACTAAAGAAGA-3′	604	[32]
	Reverse	5′-TTGCCATCCTTCTCAAAGTTGTAGG-3′		
MMP-9	Forward	5′-CACTGTCCACCCCTCAGAGC-3′	263	[32]
	Reverse	5′-GCCACTTGTCGGCGATAAGG-3′		
TIMP-1	Forward	5′-ATCCTGTTGTTGCTGTGGCTGATAG-3′	689	[32]
	Reverse	5′-TGCTGGGTGGTAACTCTTTATTTCA-3′		
TIPM-2	Forward	5′-AAACGACATTTATGGCAACCCTAT-3′	405	[32]
	Reverse	5′-ACAGGAGCCGTCACTTCTCTTGATG-3′		
Ost	Forward	5′-CTCACACTCCTCGCCCTATT-3′	165	[40]
	Reverse	5′-GACTGGGGCTCCCAGCCATT-3′		
GAPDH	Forward	5′-ATGCAACGGATTTGGTCGTAT-3′	220	[33]
	Reverse	5′-TCTCGCTCCTGGAAGATGGTG-3′		

be filled with cells and fluid. The CollaTape® scaffolds were used as carriers for osteoblasts.

Osteoblast culture in the presence of BMPs

Osteoblasts were detached from 75 cm^2 culture flasks using trypsin. They were washed twice in culture medium and counted. Cells were seeded onto CollaTape® at a density of 1×10^4 cm^{-2}. After 24 h, the CollaTape® cultures were supplemented with BMP-2 (Medtronic Ltd, Mississauga, ON, Canada) or BMP-7 (Prospec Tany TechnoGene Ltd, Rehovot, Israel) or a mixture of BMP-2/BMP-7 at final concentrations of 10 ng ml^{-1} or 100 ng ml^{-1}. The cultures were incubated in a humid 5% CO_2 atmosphere at 37 °C for different periods. The culture medium was replaced every 48 h with fresh medium with or without BMP-2, BMP-7 or a mixture of BMP-2/BMP-7. After 4 days, the culture supernatants and osteoblast-populated scaffolds were used for various analyses.

Osteoblast visualization by Hoechst staining

CollaTape® scaffolds are not optically transparent. Hoechst staining was thus used to visualize the cells attached to the scaffolds. Osteoblast-populated scaffolds were washed three times with PBS and incubated with Hoechst stain (1 μg ml^{-1}) (Molecular Probes, Eugene, OR, USA) at room temperature for 15 min. They were washed again and mounted with a cover slip in 50% glycerol mounting medium. The stained cells were observed and photographed with an epifluorescence microscope (Axiophot, Zeiss, Oberkochen, Germany).

MTT assay to assess the effect of BMPs on osteoblast growth in the scaffolds

The growth of BMP-stimulated and unstimulated osteoblasts was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Sigma, St Louis, MO, USA), which measures cell growth as a function of mitochondrial activity according to Denizot and Lang [29]. It is based on the hydrolysis of the tetrazolium ring by mitochondrial dehydrogenase, resulting in an insoluble blue reaction product (formazan). Briefly, a stock solution (5 mg ml^{-1}) of MTT was prepared in PBS and added to each culture well at a final concentration of 1% (v/v). BMP-stimulated and unstimulated osteoblast cultures were incubated for 4 h at 37 °C with MTT. The supernatant was then removed and 2 ml of 0.04 N HCl in isopropanol was added to the culture wells, and the incubation was extended for another 15 min. Lastly, 200 μl (in triplicate) of the reaction mixtures was transferred to the wells of a 96-flat-bottom plate, and the absorbance was measured at 550 nm using an enzyme-linked immunosorbent assay (ELISA) reader (model 680, BioRad Laboratories, Mississauga, ON, Canada). Results are reported as the means ± SD of eight separate experiments.

RT-PCR analyses to assess the effect of BMPs on osteocalcin, IL-6, MMP and TIMP gene activation

Following BMP stimulation, total cellular RNA was prepared from MG-63 osteoblast-like cells using Qiagen RNeasy Mini kits (Qiagen, Valencia, CA, USA). The mRNA was reverse transcribed into cDNA as previously reported [30–33]. Two microlitres of each cDNA product was added to 24 μl of PCR mixture containing Taq polymerase (Qiagen) and forward and reverse specific primers (Biosource, Montreal, QC, Canada) (table 1) [22–25]. All reactions were performed in a Perkin-Elmer Cetus DNA thermal cycler (Perkin-Elmer Cetus, Morwalk, CT, USA) as previously reported [30–33]. The RT-PCR conditions were as follows: IL-1β: 95 °C for 5 min, 35 cycles at 94 °C for 1 min, 59 °C for 45 s, 72 °C for 1 min and a final extension at 72 °C for 10 min; IL-6: 95 °C for 2 min, 35 cycles at 94 °C for 1 min, 59 °C for 45 s, 72 °C for 1 min and a final extension at 72 °C for 10 min; MMP-2 and TIMP-1: 95 °C for 2 min, 30 cycles at 95 °C for 1 min, 56 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 10 min; TIMP-2: 95 °C for 2 min, 35 cycles at 95 °C for

1 min, 56 °C for 30 s, 72 °C for 1 min and a final extension at 72 °C for 10 min; MMP-9: 95 °C for 2 min, 37 cycles at 95 °C for 45 s, 60 °C for 1 min, 72 °C for 1 min and a final extension at 72 °C for 10 min and osteocalcin (Ost): 96 °C for 3 min, 40 cycles at 95 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s and a final extension at 72 °C for 5 min. After the PCR, 2–4 μl samples were separated on 1.5–2% agarose gels containing ethidium bromide. The gels were photographed under UV light and the relative intensities of the bands were measured on digitized images using the public domain NIH Image program.

Measurement of osteocalcin, IL-6, MMP-2, MMP-9, TIMP-1 and TIMP-2 secreted by osteoblasts following stimulation with BMPs

The concentrations of secreted Ost, IL-6, MMP-2, MMP-9, TIMP-1 and TIMP-2 were assayed in supernatants collected from unstimulated and BMP-stimulated osteoclast cultures. Measurements were performed on triplicate samples using an enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN, USA) for all molecules except Ost (Biosource Europe, S.A., Nivelles, Belgium). Supernatants were filtered through 0.22 μm filters and used to quantify Ost, IL-6, MMP-2, MMP-9, TIMP-1 and TIMP-2 concentrations according to the manufacturer's instructions. Plates were read at 450 nm and analysed using a Model 680 Microplate Reader (Bio-Rad, Hercules, CA, USA). According to the manufacturer, the ELISA kits can detect less than 0.7 pg ml^{-1} of IL-6, 0.16 ng ml^{-1} of MMP-2, 0.156 ng ml^{-1} of MMP-9, 0.08 ng ml^{-1} of TIMP-1, 0.011 ng ml^{-1} of TIMP-2 and 0.4 ng ml^{-1} of Ost. The assays were repeated four times, and the means ± SD were calculated and plotted.

Statistical analysis

All experiments were performed at least three times. Experimental values are given as means ± SD. The statistical significance of differences between the control and test values was evaluated using the ANOVA paired *t*-test. Results were considered significant at $p < 0.05$. Data were analysed using the SAS version 8.2 statistical package (SAS Institute Inc., Cary, NC, USA).

Results

BMP-2, BMP-7 and a mixture of BMP-2/BMP-7 promote osteoblast adhesion to and proliferation in the collagen scaffold

A number of studies have shown that BMPs coupled to collagenous and non-collagenous carriers can enhance bone regeneration [27, 28]. However, the impact of a combination of osteoblasts, scaffolds and BMPs on bone regeneration remains to be studied. In this context, we investigated the involvement of BMP-2, BMP-7 and a mixture of BMP-2/BMP-7 on osteoblast adhesion to and distribution and growth on a collagenous carrier. The CollaTape® scaffold was non-toxic for *in vitro* osteoblast cultures (figure 1). The

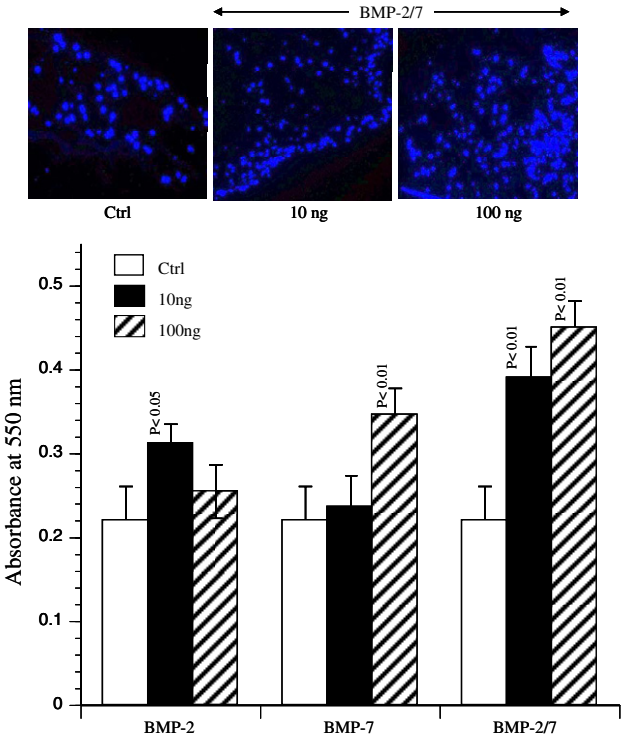

Figure 1. Effect of BMP-2 and BMP-7 on osteoblast distribution and growth. Osteoblasts were seeded onto a collagen scaffold and cultured in a humid 5% CO_2 atmosphere at 37 °C. After 24 h, the cells were stimulated with BMP-2, BMP-7 or a mixture of BMP-2/BMP-7. The BMPs were used at 10 and 100 ng ml^{-1}. Four days later, the cultures were used for Hoechst staining or for an MTT assay. The photographs (200× magnification) are representative of six separate experiments. The histograms were created using the mean relative values ± SD for six separate experiments. Differences in osteoblast growth in the presence of BMPs compared to cell growth in the absence of BMPs were considered significant at $p < 0.05$ or less.

(This figure is in colour only in the electronic version)

cells spread throughout the scaffold. The addition of BMPs to the osteoblast cultures did not inhibit cell adhesion to or proliferation in the scaffold. Hoechst staining (figure 1) revealed that BMP-stimulated cultures grew to a higher cell density than unstimulated cultures. Osteoblast growth was enhanced by BMPs. MTT analyses showed that 10 ng ml^{-1} BMP-2 ($p < 0.05$) and 100 ng ml^{-1} BMP-7 ($p < 0.01$) significantly stimulated cell growth (figure 1). While the mixture of BMP-2/BMP-7 stimulated osteoblast growth more than BMP-2 or BMP-7 (figure 1), the homodimers and their mixture all promoted osteoblast adhesion to the collagenous carrier as well as osteoblast growth.

BMPs promote osteocalcin mRNA expression and osteocalcin secretion

Osteocalcin is a non-collagenous protein present in bone and dentin. It is secreted by osteoblasts, which play an active role in bone mineralization [34]. Osteocalcin is often used as a marker for the bone formation process. Since BMPs

may play an active role in bone formation, we investigated the effect of BMP-2, BMP-7 and a mixture of BMP-2/BMP-7 on osteocalcin mRNA expression. Unstimulated osteoblasts expressed a basal level of osteocalcin (figure 2(A)). Upon stimulation with BMP-2 (100 ng ml^{-1}), osteocalcin mRNA expression increased significantly ($p < 0.05$). However, no significant effect was observed with BMP-7. Interestingly, when osteoblasts were stimulated with a mixture of BMP-2/BMP-7, osteocalcin mRNA expression increased significantly (10 ng ml^{-1}, $p < 0.05$; 100 ng ml^{-1}, $p < 0.01$). To determine whether the effect of BMPs on the stimulation of osteocalcin mRNA expression was reflected in an increased secretion of osteocalcin, we measured osteocalcin levels in the culture supernatants and found that osteoblasts constitutively secreted approximately 32 ng ml^{-1} of osteocalcin (figure 2(B)). Upon BMP-2 stimulation, twice as much osteocalcin was secreted by BMP-2-stimulated cells as by unstimulated cells. Osteocalcin concentrations increased to 60 ng ml^{-1} ($p < 0.02$) and 65 ng ml^{-1} ($p < 0.01$) upon stimulation with 10 and 100 ng ml^{-1} of BMP-2, respectively. Similar increases in osteocalcin concentrations were obtained upon BMP-7 stimulation ($p < 0.02$; $p < 0.04$). The stimulatory effect of BMP-2 on osteocalcin secretion was greater than that of BMP-7. The mixture of BMP-2/BMP-7 (10 and 100 ng ml^{-1}) also significantly increased the secretion of osteocalcin ($p < 0.01$). Interestingly, the upregulatory effect of the homodimer mixture was greater than that obtained with the homodimers (figure 2(B)). Overall, these results show that BMP-2 and BMP-7 upregulated osteocalcin gene activation and promoted osteocalcin secretion.

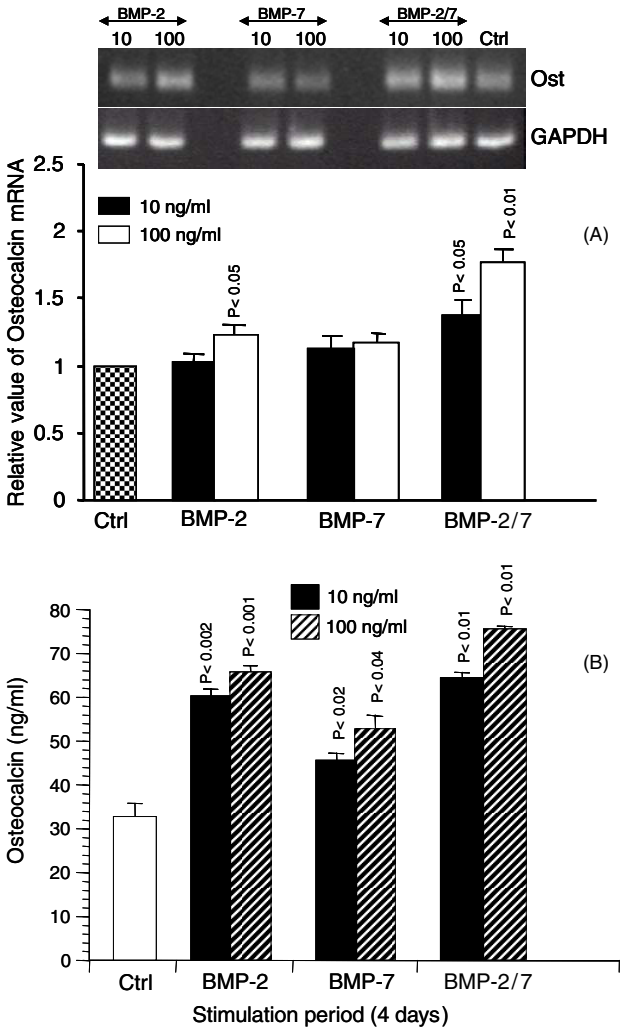

Figure 2. BMP-2 and BMP-7 modulate osteocalcin mRNA and protein expression. (A) Following osteoblast culture in the presence or absence of BMP-2 and BMP-7 homodimers and a mixture of BMP-2/BMP-7 homodimers for 4 days, total RNA was extracted from the collagen carrier and RT-PCRs were performed using specific primers for osteocalcin and GAPDH. Changes in mRNA levels were measured using the public domain NIH Image software package. BMPs stimulated cultures were compared to non-stimulated culture (ctrl). Ctrl represented 100% gene activation and was used to determine the relative expression levels of osteocalcin mRNA. Photos are representative gel of three separate experiments. Histograms are the means ± SD of three separate experiments. (B) Following BMP stimulation, cultured supernatants were collected and used to quantify osteocalcin. Culture medium was filtered through 0.22 μm filters and used to determine the amount of the secreted osteocalcin using ELISA kits. Data are the means ± SD of four separate experiments. The levels of significance were obtained by comparing the value obtained with BMP-stimulated tissue to the value obtained with BMP non-stimulated tissue.

BMP-2 and BMP-7 stimulate IL-6 but not IL-1β mRNA expression and modulate IL-6 secretion

Scaffold-grown osteoblasts were stimulated with BMP-2, BMP-7 and a mixture of BMP-2/BMP-7 for 4 days. Total mRNA was extracted and used to determine the levels of IL-1β and IL-6 mRNA. RT-PCR analyses showed no significant effect of the BMP homodimers or the mixture of BMP homodimers on IL-1β mRNA expression (data not shown). However, BMP-7 (100 ng ml^{-1}) and a mixture of BMP-2/BMP-7 (100 ng ml^{-1}) both stimulated IL-6 mRNA expression to a significant degree ($p < 0.05$ and $p < 0.01$, respectively) (figure 3(A)).

Since BMPs modulate IL-6 mRNA expression, we looked at whether this was paralleled by an increase in IL-6 secretion. ELISA analyses showed that significant amounts of IL-6 were present in the supernatants of osteoblast cultures that had been stimulated with 100 ng ml^{-1} of BMP-7 and 10 and 100 ng ml^{-1} of the mixture of BMP-2/BMP-7 (figure 3(B)). In addition, the amount of IL-6 secreted rose in parallel with the concentration of the mixture of BMP-2/BMP-7 homodimers.

BMPs modulate metalloproteinase mRNA expression and protein secretion

MMPs play an important role in maintaining extracellular matrix homeostasis. As such, we investigated the effect of BMPs on MMP-2 and MMP-9 mRNA expressions. Osteoblasts expressed a high basal level of MMP-2 mRNA, which was not significantly modulated following stimulation BMP-2 or BMP-7 (figure 4(A)). However, the mixture of BMP-2/BMP-7 (10 ng ml^{-1}) caused a significant ($p < 0.03$) decrease in MMP-2 mRNA expression. The homodimers and

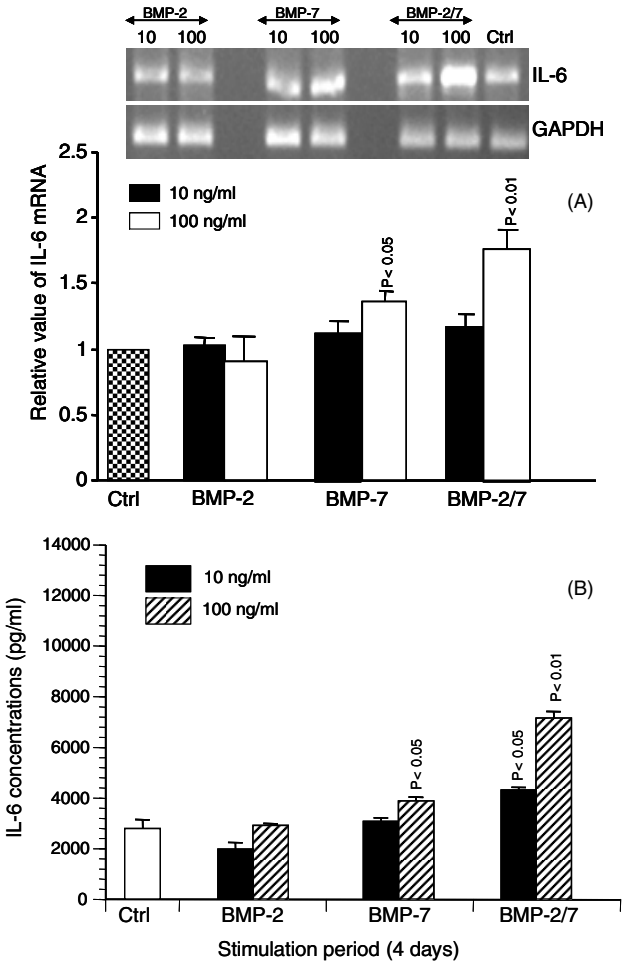

Figure 3. BMP-2 and BMP-7 modulate IL-6 mRNA and protein expression by osteoblasts. (A) Following osteoblast culture in the presence or absence of BMP-2 and BMP-7 homodimers and a mixture of BMP-2/BMP-7 homodimers for 4 days, total RNA was extracted from the collagen carrier and RT-PCRs were performed using specific primers for IL-6 and GAPDH. Changes in mRNA levels were measured using the public domain NIH Image software package. BMP-stimulated cultures were compared to non-stimulated culture (ctrl). Ctrl represented 100% gene activation and was used to determine the relative expression levels of IL-6 mRNA. Photos are representative gel of three separate experiments. Histograms are mean ± SD of three separate experiments. (B) Following BMP stimulation, cultured supernatants were collected and used to quantify IL-6. Culture medium was filtered through 0.22 μm filters and used to determine the amount of the secreted IL-6 using ELISA kits. Data are the means ± SD of four separate experiments. The levels of significance were obtained by comparing the value obtained with BMP-stimulated tissue to the value obtained with BMP non-stimulated tissue.

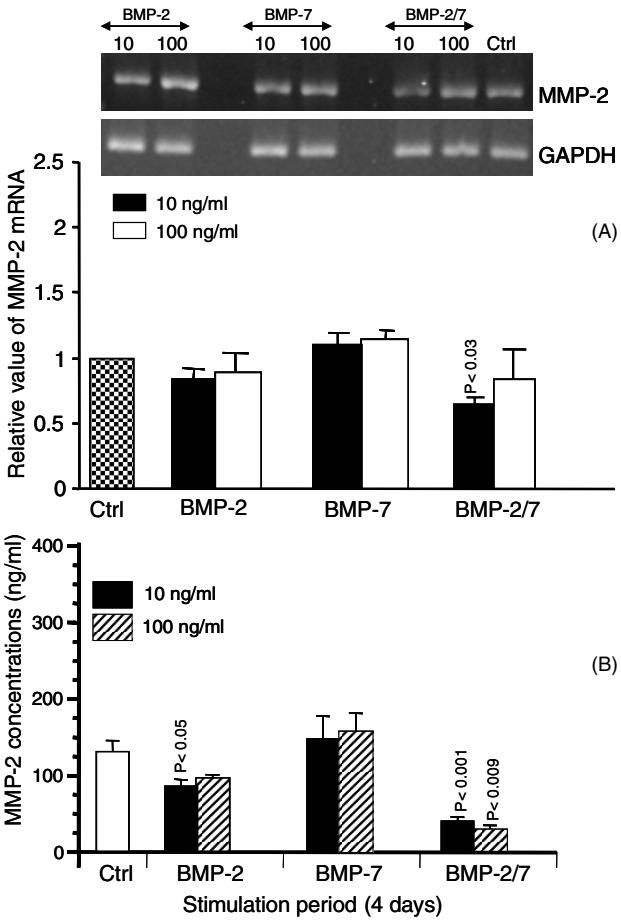

Figure 4. BMP-2 and BMP-7 downregulated MMP-2 mRNA and protein expression. (A) Following osteoblast culture in the presence or absence of BMP-2 and BMP-7 homodimers and a mixture of BMP-2/BMP-7 homodimers for 4 days, total RNA was extracted from the collagen carrier and RT-PCRs were performed using specific primers for MMP-2 and GAPDH. Changes in mRNA levels were measured using the public domain NIH Image software package. BMP-stimulated cultures were compared to non-stimulated culture (ctrl). Ctrl represented 100% gene activation and was used to determine the relative expression levels of MMP-2 mRNA. Photos are representative gel of three separate experiments. Histograms are mean ± SD of three separate experiments. (B) Following BMP stimulation, cultured supernatants were collected and used to quantify MMP-2. Culture medium was filtered through 0.22 μm filters, and used to determine the amount of the secreted MMP-2 using ELISA kits. Data are the means ± SD of four separate experiments. The levels of significance were obtained by comparing the value obtained with BMP-stimulated tissue to the value obtained with BMP non-stimulated tissue.

their mixture had no effect on MMP-9 mRNA expression (data not shown). Four day cultures of unstimulated osteoblasts constitutively secreted up to 120 ng ml^{-1} of MMP-2. BMP-2 (10 ng ml^{-1}) significantly inhibited the secretion of MMP-2 by osteoblasts ($p < 0.05$) while BMP-7 had no significant effect. On the other hand, the mixture of BMP-2/BMP-7 homodimers significantly decreased the amount of secreted MMP-2 from the 120 ng ml^{-1} basal level to 45 ng ml^{-1} with 10 ng ml^{-1} of the mixture of BMP-2/BMP-7 and to 35 ng ml^{-1} with 100 ng ml^{-1} of the mixture of BMP-2/BMP-7 (figure 4(B)). The BMP homodimers and their mixture had no significant effect on MMP-9 secretion (data not shown). These results showed that BMP-2 and the mixture of BMP-2/BMP-7 modulated MMP-2 secretion, which confirmed their effect on MMP-2 mRNA expression.

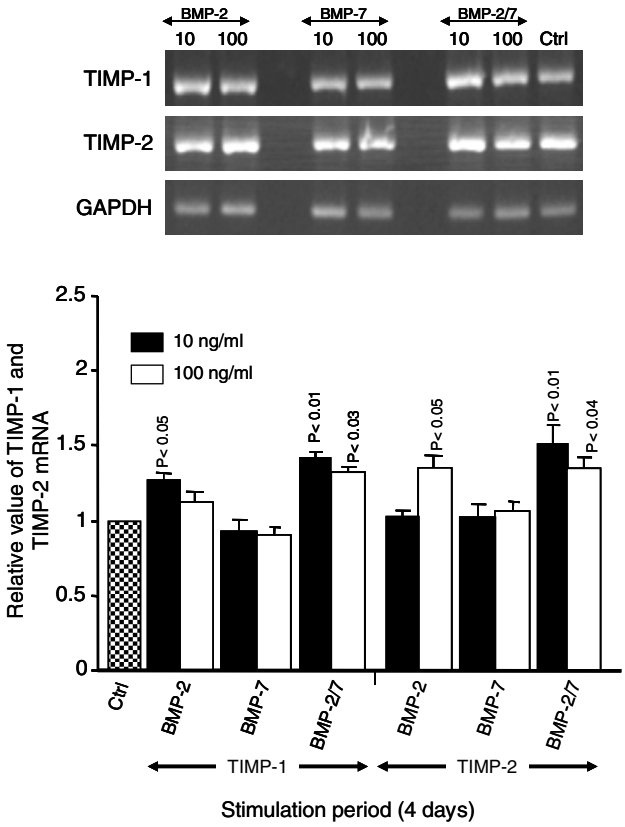

Figure 5. BMP-2 and BMP-7 upregulated TIMP-1 and TIMP-2 mRNA expressions. Following osteoblast culture in the presence or absence of BMP-2 and BMP-7 homodimers and a mixture of BMP-2/BMP-7 homodimers, total RNA was extracted and RT-PCRs were performed using specific primers for TIMP-1, TIMP-2 and GAPDH. Changes in mRNA levels were measured using the public domain NIH Image software package. BMP-stimulated cultures were compared to non-stimulated culture (ctrl). Ctrl represented 100% gene activation and was used to determine the relative expression levels of TIMP-1 and TIMP-2 mRNA. Photos are representative gel of three experiments. Histograms are mean ± SD of four experiments. The statistical differences were obtained by comparing the value obtained with the BMP-stimulated tissue to the value obtained with the BMP non-stimulated tissue.

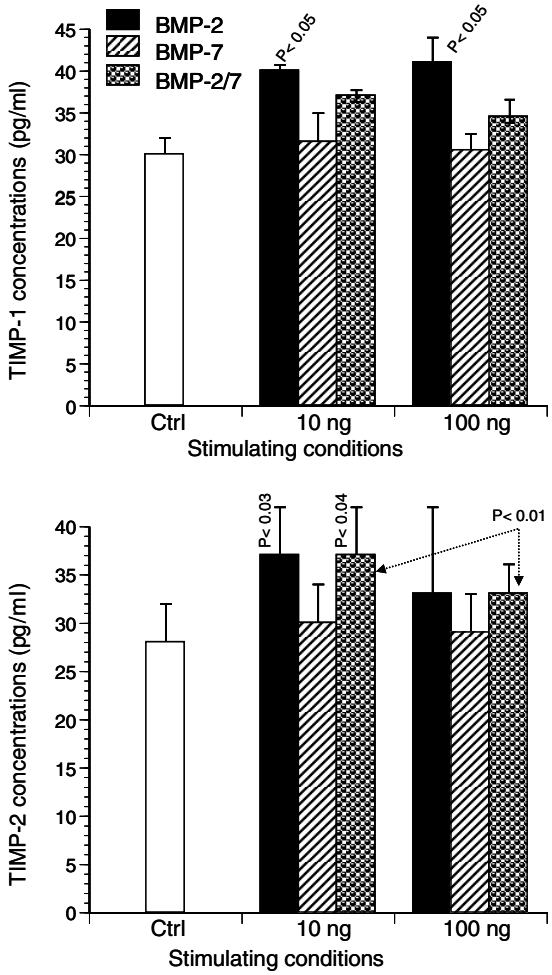

Figure 6. BMP-2 and BMP-7 increased TIMP-1 and TIMP-2 secretions. Following osteoblast culture into a collagen scaffold in the presence or absence of BMP-2 and BMP-7 homodimers and a mixture of BMP-2/BMP-7 homodimers, cultured supernatants were collected and used to quantify metalloproteinase inhibitors. Culture medium was filtered through 0.22 μm filters and used to determine the amount of secreted protease inhibitors using ELISA kits. Data are the means ± SD of three separate experiments. The levels of significance were obtained by comparing the value obtained with BMP-stimulated tissue to the value obtained with BMP non-stimulated tissue.

BMPs modulate TIMP mRNA expression and protein secretion

The production/degradation activities of MMPs are regulated by TIMPs. Since MMP-2 mRNA expression and protein secretion was modulated by BMPs, we looked at the effect of the BMPs on TIMP mRNA expression and TIMP secretion. TIMP-1 and TIMP-2 mRNA expressions were modulated by BMPs (figure 5). Both homodimers had a significant ($p < 0.05$) stimulatory effect on TIMP-1 and TIMP-2 mRNA expressions as compared to the control (non-stimulated cultures). The mixture of BMP-2/BMP-7 increased TIMP-1 and TIMP-2 mRNA expressions much more than BMP-2. To determine whether the effects of the BMPs on TIMP mRNA expression resulted in more protein secretion, TIMP-1 and TIMP-2 concentrations in the culture supernatants were measured. While the largest increase in TIMP-1 secretion was obtained with 10 and 100 ng ml^{-1} BMP-2 (figure 6), both the homodimers and their mixture caused a significant increase in TIMP-1 secretion by osteoblasts. On the other hand, BMP-2 (10 ng ml^{-1}) significantly increased ($p < 0.03$) the secretion of TIMP-2 while BMP-7 had no effect. The secretion of TIMP-2 was significantly increased by 10 ng ml^{-1} of the mixture of BMP-2/BMP-7 ($p < 0.04$) but decreased in the presence of 100 ng ml^{-1} of the mixture of BMP-2/BMP-7.

Discussion

Growth factors and bioactive molecules released by various cells, as well as locally secreted BMPs, play a role in bone regeneration through cell migration and proliferation

[35]. BMP homodimers and heterodimers contribute to osteogenic differentiation and bone formation [13, 14]. In the present study, we showed that BMP-2, BMP-7 and a mixture of BMP-2/BMP-7 promote osteoblast adhesion to and growth on a collagen scaffold. The scaffold mimicked the *in vivo* physiological conditions of the ECM. The scaffold also acted as a carrier for the BMPs. Previous reports have shown that natural and synthetic scaffolds trap BMPs and deliver them locally following implantation of the carriers. This process initiates and promotes bone regeneration [12]. Using the engineered bone model created using CollaTape®, we showed that BMP-2, BMP-7 and a mixture of BMP-2/BMP-7 promoted osteoblast adhesion and proliferation, which is in agreement with previously reported results [14]. We also demonstrated that the mixture of homodimers was more effective than the homodimers in promoting osteoblast adhesion and proliferation. This supports previously published reports [13, 36] indicating that different BMPs can act synergistically to enhance bone regeneration. The positive effect of BMPs on cell growth and bone regeneration can be achieved with low [14] or high [37] concentrations, depending on the experimental model used. In our engineered bone model, osteoblast growth was promoted by low BMP concentrations (10 and 100 ng ml^{-1}). This is in agreement with other studies showing that lower concentrations of rhBMP-7 than those of rhBMP-2 are required to promote intramuscular ectopic bone formation [22]. BMPs promote osteogenesis by osteoblasts as well as by pluripotent mesenchymal cells. They also stimulate the expression of cbfa-1 [38], an important transcription factor for osteoblast differentiation and bone formation. Osteocalcin expression is associated with osteoblast mineralization and may be a terminal marker of cell differentiation [34]. The increase in osteocalcin mRNA expression and protein secretion reported here confirms the involvement of BMP-2 and BMP-7 in bone regeneration and is in agreement with the findings of Chen *et al*, who showed that osteocalcin gene activation is promoted by BMP-2 [39]. However, BMP-2 promoted bone regeneration greater than BMP-7 as ascertained by the level of osteocalcin gene activation and protein production. The higher effect of BMP-2 compared to BMP-7 on bone formation we showed in the present work confirmed results reported by Kang *et al*, which indicate that the effects of rhBMP-2 on bone formation are more potent than rhBMP-4 or rhBMP-7 [19].

IL-6 may play an important role in bone regeneration through the regulation of osteoclast and osteoblast development and function [40]. Our finding that BMP-7 and a mixture of BMP-2/BMP-7 upregulated IL-6 mRNA expression and protein secretion provides support for this suggestion. IL-6 may be linked to osteocalcin secretion since it has been reported that IL-6, combined with its receptor, increases osteocalcin mRNA expression and the formation of mineralizing collagen fibres [41]. This could be another pathway leading to osteocalcin production independent of BMP-2, because the morphogenic protein did not modulate IL-6 secretion by osteoblasts. The expression and secretion of osteocalcin reflect the maturation status of osteoblasts [42]. Mature osteoblasts synthesize and secrete ECM, with collagen constituting a major part of the ECM. BMP-2 enhances type I collagen mRNA expression [43]. The production of ECM proteins is regulated by various proteolytic enzymes, including metalloproteinases (MMPs) [43]. The involvement of MMPs in the ECM production/degradation equilibrium is well documented [43]. To our knowledge, our results show for the first time that BMP-2 and a mixture of BMP-2/BMP-7 downregulate MMP-2 mRNA expression and protein secretion and increase TIMP mRNA expression and protein secretion, suggesting that BMPs are involved in the regulation of ECM production by osteoblasts. This is consistent with previously reported studies on BMPs, collagenase-3 and their inhibitors [44, 45]. Our results demonstrate that BMP-2 and a mixture of BMP-2/BMP-7 are involved in ECM production by osteoblasts through an inhibitory effect on the expression and secretion of MMPs. Further study is required to understand the specific involvement of BMPs on bone regeneration. One question that still needs an answer is: Why does a low concentration (10 ng ml^{-1}) of BMP-2/BMP-7 decrease MMP-2 gene expression but not 100 ng ml^{-1} of this mixture?

In summary, BMP homodimers (BMP-2 and BMP-7) and their mixture (BMP-2/BMP-7) promoted osteoblast adhesion and growth. They also modulated osteocalcin secretion, possibly through an IL-6 pathway as previously suggested [41]. BMP-2, BMP-7 and the mixture of BMP-2/BMP-7 may promote ECM production by inhibiting MMP secretion through a TIMP pathway. Our results point to the involvement of BMP-2 and BMP-7 in bone regeneration and confirm their potential use for clinical applications.

Acknowledgment

This study was supported by operating grants from the Fonds Émile-Beaulieu, FMD.

References

[1] Boyne P J 2001 Application of bone morphogenetic proteins in the treatment of clinical oral and maxillofacial osseous defects *J. Bone Joint Surg. Am.* **83A** S146–50
[2] Geiger M, Li R H and Friess W 2003 Collagen sponges for bone regeneration with rhBMP-2 *Adv. Drug Deliv. Rev.* **55** 1613–29
[3] Carlisle E and Fischgrund J S 2005 Bone morphogenetic proteins for spinal fusion *Spine J.* **5** S240–9
[4] Kenley R A, Yim K, Abrams J, Ron E, Turek T, Marden L J and Hollinger J O 1993 Biotechnology and bone graft substitutes *Pharm. Res* **10** 1393–401
[5] Hollinger J O, Uludag J O and Winn S R 1998 Sustained release emphasizing recombinant human bone morphogenetic protein-2 *Adv. Drug Deliv. Rev.* **31** 303–18
[6] Artico M, Ferrante L, Pastore F S, Ramundo E O, Cantarelli D, Scopelliti D and Iannetti G 2003 Bone autografting of the calvaria and craniofacial skeleton: historical background, surgical results in a series of 15 patients, and review of the literature *Surg. Neurol* **60** 71–9
[7] Giannoudis P V, Dinopoulos H and Tsiridis E 2005 Bone substitutes: an update *Injury* **36** Suppl. 3 S20–7
[8] Eppley B L, Pietrzak W S and Blanton M W 2005 Allograft and alloplastic bone substitutes: a review of science and technology for the craniomaxillofacial surgeon *J. Craniofac. Surg.* **16** 981–9

[9] Dimitriou R and Giannoudis P V 2005 Discovery and development of BMPs *Injury* **36** Suppl. 3 S28–33
[10] Groeneveld E H and Burger E H 2000 Bone morphogenetic proteins in human bone regeneration *Eur. J. Endocrinol.* **142** 9–21
[11] Nakagawa T and Tagawa T 2000 Ultrastructural study of direct bone formation induced by BMPs-collagen complex implanted into an ectopic site *Oral Dis.* **6** 172–9
[12] Kato M, Toyoda H, Namikawa T, Hoshino M, Terai H, Miyamoto S and Takaoka K 2006 Optimized use of a biodegradable polymer as a carrier material for the local delivery of recombinant human bone morphogenetic protein-2 (rhBMP-2) *Biomaterials* **27** 2035–41
[13] Israel D I, Nove J, Kerns K M, Kaufman R J, Rosen V, Cox K A and Wozney J M 1996 Heterodimeric bone morphogenetic proteins show enhanced activity *in vitro* and *in vivo Growth Factors* **13** 291–300
[14] Zhu W, Rawlins B A, Boachie-Adjei O, Myers E R, Arimizu J, Choi E, Lieberman J R, Crystal R G and Hidaka C 2004 Combined bone morphogenetic protein-2 and -7 gene transfer enhances osteoblastic differentiation and spine fusion in a rodent model *J. Bone Miner. Res.* **19** 2021–32
[15] Bostrom M P, Lane J M, Berberian W S, Missri A A, Tomin E, Weiland A, Doty S B, Glaser D and Rosen V M 1995 Immunolocalization and expression of bone morphogenetic proteins 2 and 4 in fracture healing *J. Orthop. Res.* **13** 357–67
[16] Onishi T, Ishidou Y, Nagamine T, Yone K, Imamura T, Kato M, Sampath T K, ten Dijke P and Sakou T 1998 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* **22** 605–12
[17] Mayer H, Scutt A M and Ankenbauer T 1996 Subtle differences in the mitogenic effects of recombinant human bone morphogenetic proteins -2 to -7 on DNA synthesis on primary bone-forming cells and identification of BMP-2/4 receptor *Calcif. Tissue Int.* **58** 249–55
[18] Hogan B L 1996 Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes Dev.* **10** 1580–94
[19] Kang Q *et al* 2004 Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery *Gene Ther.* **11** 1312–20
[20] Peng Y, Kang Q, Cheng H, Li X, Sun M H, Jiang W, Luu H H, Park J Y, Haydon R C and He T C 2003 Transcriptional characterization of bone morphogenetic proteins (BMPs)-mediated osteogenic signalling *J. Cell. Biochem.* **90** 1149–65
[21] de Jong D S, Vaes B L, Dechering K J, Feijen A, Hendriks J M, Wehrens R, Mummery C L, van Zoelen E J, Olijve W and Steegenga W T 2004 Identification of novel regulators associated with early-phase osteoblast differentiation *J. Bone Miner. Res.* **19** 947–58
[22] Aono A, Hazama M, Notoya K, Taketomi S, Yamasaki H, Tsukuda R, Sasaki S and Fujisawa Y 1995 Potent ectopic bone-inducing activity of bone morphogenetic protein-4/7 heterodimer *Biochem. Biophys. Res. Commun.* **210** 670–7
[23] Lyons K M, Hogan B L and Robertson E J 1995 Colocalization of BMP-7 and BMP-2 RNAs suggests that these factors cooperatively mediate tissue interactions during murine development *Mech. Dev.* **50** 71–83
[24] Suzuki Y, Tanihara M, Suzuki K, Saitou A, Sufan W and Nishimura Y 2000 Alginate hydrogel linked with synthetic oligopeptide derived from BMP-2 allows ectopic osteoinduction *in vivo J. Biomed. Mater. Res.* **50** 405–9
[25] Giannoudis P V and Tzioupis C 2005 Clinical applications of BMP-7: the UK perspective *Injury* **36** Suppl. 3 S47–50
[26] Brown A, Stock G, Patel A A, Okafor C and Vaccaro A 2006 Osteogenic protein-1: a review of its utility in spinal applications *Biodrugs* **20** 243–51
[27] Saito N and Takaoka K 2003 New synthetic biodegradable polymers as BMP carriers for bone tissue engineering *Biomaterials* **24** 2287–93
[28] Liu H W, Chen C H, Tsai C L and Hsiue G H 2006 Targeted delivery system for juxtacrine signaling growth factor based on rhBMP-2-mediated carrier-protein conjugation *Bone* **39** 825–36
[29] Denizot F and Lang R 1986 Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability *J. Immunol. Methods* **89** 271–7
[30] Tardif F, Ross G and Rouabhia M 2004 Gingival and dermal fibroblasts produce interleukin-1 beta converting enzyme and interleukin-1 beta but not interleukin-18 even after stimulation with lipopolysaccharide *J. Cell. Physiol.* **198** 125–32
[31] Mostefaoui Y, Bart C, Frenette M and Rouabhia M 2004 *Candida albicans* and *Streptococcus salivarius* modulate IL-6, IL-8, and TNF-alpha expression and secretion by engineered human oral mucosa cells *Cell. Microbiol.* **6** 1085–96
[32] Claveau I, Mostefaoui Y and Rouabhia M 2004 Basement membrane protein and matrix metalloproteinase deregulation in engineered human oral mucosa following infection with *Candida albicans Matrix Biol.* **23** 477–86
[33] Rouabhia M, Ross G, Page N and Chakir J 2002 Interleukin-18 and gamma interferon production by oral epithelial cells in response to exposure to *Candida albicans* or lipopolysaccharide stimulation *Infect. Immun.* **70** 7073–80
[34] Desbois C and Karsenty G 1995 Osteocalcin cluster: implications for functional studies *J. Cell. Biochem.* **57** 379–83
[35] Albrektsson T and Johansson C 2001 Osteoinduction, osteoconduction and osseointegration *Eur. Spine J.* **10** Suppl. 2 S96–101
[36] Aoki H, Fujii M, Imamura T, Yagi K, Takehara K, Kato M and Miyazono K 2001 Synergistic effects of different bone morphogenetic protein type I receptors on alkaline phosphatase induction *J. Cell. Sci.* **114** 1483–9
[37] Sampath T K, Coughlin J E, Whetstone R M, Banach D, Corbett C, Ridge R J, Ozkaynak E, Oppermann H and Rueger D C 1990 Bovine osteogenic protein is composed of dimers of OP-1 and BMP-2A, two members of the transforming growth factor-beta superfamily *J. Biol. Chem.* **265** 13198–205
[38] Tsuji K, Ito Y and Noda M 1998 Expression of the PEBP2alphaA/AML3/CBFA1 gene is regulated by BMP4/7 heterodimer and its overexpression suppresses type I collagen and osteocalcin gene expression in osteoblastic and nonosteoblastic mesenchymal cells *Bone* **22** 87–92
[39] Chen D, Harris M A, Rossini G, Dunstan C R, Dallas S L, Feng J Q, Mundy G R and Harris S E 1997 Bone morphogenetic protein 2 (BMP-2) enhances BMP-3, BMP-4, and bone cell differentiation marker gene expression during the induction of mineralized bone matrix formation in cultures of fetal rat calvarial osteoblasts *Calcif. Tissue Int.* **60** 283–90
[40] Manolagas S C 1998 The role of IL-6 type cytokines and their receptors in bone *Ann. NY Acad. Sci.* **840** 194–204
[41] Taguchi Y, Yamamoto M, Yamate T, Lin S C, Mocharla H, DeTogni P, Nakayama N, Boyce B F, Abe E and Manolagas S C 1998 Interleukin-6-type cytokines stimulate mesenchymal progenitor differentiation toward the osteoblastic lineage *Proc. Assoc. Am. Physicians* **110** 559–74

[42] Thies R S, Bauduy M, Ashton B A, Kurtzberg L, Wozney J M and Rosen V 1992 Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20-17 stromal cells *Endocrinology* **130** 1318–24

[43] Sternlicht M D and Werb Z 2001 How matrix metalloproteinases regulate cell behavior *Annu. Rev. Cell Dev. Biol.* **17** 463–516

[44] Gazzerro E, Rydziel S and Canalis E 1999 Skeletal bone morphogenetic proteins suppress the expression of collagenase-3 by rat osteoblasts *Endocrinology* **140** 562–7

[45] Varghese S and Canalis E 1997 Regulation of collagenase-3 by bone morphogenetic protein-2 in bone cell cultures *Endocrinology* **138** 1035–40