J Mater Sci: Mater Med (2007) 18:255–263
DOI 10.1007/s10856-006-0687-4

Differentiation of preosteoblasts using a delivery system with BMPs and bioactive glass microspheres

E. Bergeron · M. E. Marquis · I. Chrétien · N. Faucheux

Received: 16 June 2006 / Accepted: 9 November 2006

Abstract Bone morphogenetic proteins (BMPs) and 45S5 Bioglass® microspheres (bioactive GM) can increase the differentiation of osteoblasts. Recombinant human BMP-2 (rhBMP-2) is presently the BMP most frequently used in delivery systems and it has already been used in clinical bone healing studies. We have developed a delivery system that combines a collagen Type I gel, BMP and bioactive GM. Since BMP-9 seems to be more osteogenic than BMP-2, we compared the differentiation of MC3T3-E1 preosteoblasts induced by our delivery system containing either a peptide derived from BMP-9 (pBMP-9), or rhBMP-2, both at 100 ng/mL. After 5 days, alkaline phosphatase staining showed that pBMP-9 induced more differentiation than rhBMP-2 in all experimental conditions. Also, bioactive GM increased this BMP effect. Since preosteoblasts secreted matrix metalloproteinases (MMPs) that can degrade collagen, we then studied the influence of the delivery system on MMPs production. We observed that MMP-2 was the major MMP involved in all experimental conditions. In addition, pBMP-9 with bioactive GM generated less MMP-2 than did rhBMP-2 on days 3 and 5. Thus, a delivery system using collagen Type I gel with pBMP-9 and bioactive GM seems to be a promising system for bone regeneration.

1 Introduction

Several growth factors, including bone morphogenetic proteins (BMP), transforming growth factor beta, fibroblast growth factor and insulin-like growth factor, stimulate bone regeneration [1]. In 1965, Urist discovered the osteoinductive properties of demineralised bone matrix due to BMPs [2]. The BMP concentration in the demineralised bone matrix was estimated to be 1–2 μg in a kg of bone [3, 4]. BMPs were subsequently cloned and recombinant proteins produced had also osteoinductive properties [1, 3].

Most clinical trials have used BMP family growth factors, especially BMP-2 [5, 6]. *In vitro*, BMP-2 regulates osteoblast differentiation [7, 8] and can also stimulate bone resorption by directly stimulating the differentiation of osteoclasts and activating mature osteoclasts [9, 10]. While BMP-2 can accelerate the healing of closed fracture in small animals like rabbits, it is much less effective in larger animals because not enough of it is retained at the repair site [11]. Pharmacokinetic studies have also demonstrated that the half life of BMP-2 in nonhuman primates is about 6.7 min [10]. Therefore, new strategies have been developed that combine BMP-2 with implanted carriers to avoid the growth factor degradation and improve its concentration at the bone repair site. BMP delivery system using carriers must be biocompatible, biodegradable, malleable, easy to handle, sterilizable and act as a stable structural support for cell colonization, proliferation and differentiation [1, 12, 13]. Implantable carriers can be made from natural polymers, such as collagen, chitosan, hyaluronic acid, and synthetic materials like poly(lactide coglycolide) [1, 12, 14–17]. Since collagen Type I is the most abundant component of the extracellular bone matrix synthesized by osteoblasts, most delivery systems used this weakly immunogenic protein [14]. Collagen can also be treated with enzymes and/or by chemical or physical treatments during preparation to control biodegradation *in vivo* and decrease its immunogenic properties [12]. Delivery systems combining BMP-2 and collagen have received USA Food and Drug Administration approval for clinical trials. For example, Infuse®

E. Bergeron · M. E. Marquis · I. Chrétien · N. Faucheux (✉)
Laboratory of Cell-Biomaterial Biohybrid Systems, Université de Sherbrooke, Chemical Engineering Department, 2500, Université Blvd, Sherbrooke, Québec, Canada, J1K 2R1
e-mail: Nathalie.Faucheux@Usherbrooke.ca

bone graft is used to treat spine degenerative diseases and the healing of tibia fractures [12, 13].

Some studies have also demonstrated the effects of composite carriers using collagen and bioactive glass microspheres (bioactive GM) on osteoblast differentiation and bone formation [18, 19]. Bioactive GM stimulate the formation of apatite at rates that depend on their porosity and formulation, especially CaO and P_2O_5 [19]. For example, 45S5 Bioglass® triggers the formation of induced apatite in half a day *in vitro* [20]. These bioactive GM can support osteoblast growth *in vitro* and favour osteoblast differentiation by stimulating the synthesis of phenotypic markers like alkaline phosphatase (ALP), collagen Type I and osteocalcin (OC) [21–24].

But, osteoblasts also secrete matrix metalloproteinases (MMPs) during their differentiation. The MMP is a large class of proteinases, whose 23 members are involved in the degradation and remodelling of the organic bone matrix [25–27]. However, few studies have analyzed the impact of a complex delivery system on the ability of cells to secrete MMP [26, 28].

We have therefore developed a delivery system combining collagen Type I, BMP and bioactive GM. MC3T3-E1 preosteoblasts were used as a model of osteogenesis *in vitro* since their developmental sequence is typical of osteoblasts; they produce marker proteins like ALP, collagen Type I and OC [8]. Similarly with numerous *in vitro* studies, we used 100 ng/mL recombinant human BMP-2 (rhBMP-2) [29–32]. Since BMP-9 increases the ALP activity of osteoblasts more than does BMP-2 [5], we have analyzed the impact of a peptide derived from BMP-9 (pBMP-9) when included in the delivery system. We first characterized the collagen Type I extracted from rat tail tendons. We then analyzed the size distribution of bioactive GM with reference to plain silicon oxide microspheres (plain GM). The third step was to determine the release of rhBMP-2 from the delivery system. Finally, we compared the influence of rhBMP-2 and pBMP-9 on preosteoblast differentiation and the secretion of MMPs by these cells.

2 Materials and methods

2.1 Cells

Murine calvarial preosteoblasts MC3T3-E1 (CRL-2594™, ATCC®, Manassas, VA, USA) were grown at 37°C in Minimum Essential Medium (MEM) alpha medium without ascorbic acid (α-MEM, Gibco®, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma, Oakville, Canada), 2 mM L-Glutamine (Invitrogen™, Burlington, ON, Canada), 100 U/mL penicillin (Gibco) and 100 μg/mL streptomycin (Gibco) under a humidified 5% CO_2 atmosphere. Cells were used for experiments between passages 3 and 12. Cells were removed by trypsinization (Invitrogen), suspended in α-MEM with 10% FBS and seeded in 24-well cell culture plates ($4 \times 10^4/cm^2$). When cells reached 90% confluence, the medium was replaced with α-MEM without FBS for one day. The medium was then replaced with 500 μL α-MEM without FBS and the delivery system was introduced.

Cell viability was determined by flow cytometer (Guava EasyCyte™ System, Guava Technologies, Hayward, CA, USA) and the Guava ViaCount® Assay kit, used as in the manufacturer's instructions. Apoptotic cells were also measured by flow cytometry analysis after staining for annexin V, a calcium-dependent phospholipid-binding protein and/or 7-amino actinomycin, which binds to membranes whose integrity has been altered (Guava Nexin™ Assay kit, Guava Technologies).

2.2 Collagen matrix

Collagen Type I was extracted from rat tail tendons in ethanol 95% and dried overnight under a UV lamp. Tendons (1 cm lengths) were dissolved by placing them in 0.5 N acetic acid at 4°C, with gentle magnetic stirring for 4 days. The resulting collagen solution was centrifuged at 30 000 g for 60 min. The supernatant containing collagen Type I was stored at −20°C. Collagen Type I was first immunoprecipitated. Commercial rat tail collagen Type I (Sigma) was used as a control. Each source of collagen (100 μg in 500 μL) was incubated with 20 μL protein G-agarose beads (Sigma) and 2 μg primary mouse antibodies directed against collagen Type I (COL-1, Sigma) for 120 min at 4°C in a tube rotator. Any non-specific binding was removed by washing three times with ice-cold phosphate-buffered saline (PBS). The washed protein-bead complex was then boiled with 50 μL NuPAGE® LDS 1× sample buffer (Invitrogen). Collagen was resolved by electrophoresis at 50 V for 5 h on 7.5% SDS-PAGE gel (Bio-Rad Laboratories, Mississauga, ON, Canada). The gels were then stained with Coomassie solution (0.25% Coomassie® Brilliant Blue G-250 (w:v); Pierce Biotechnology, Rockford, IL, USA), 40% (v/v) methanol and 10% (v/v) acetic acid) for 30 min. Then, they were destained three times for 10 min in aqueous 40% (v/v) methanol, 10% (v/v) acetic acid and dried in Gel-Dry™ Drying Solution (Invitrogen). The purity of the extracted collagen from rat tail tendons was then determined by dot blot analysis. Commercial collagen was again used as a control. Aliquots (4 × 10 μL) of each collagen solution were applied to a porous (0.45 μm) nitrocellulose membrane (Bio-Rad Laboratories). Non-specific binding sites on the membrane were blocked by incubating it with 5% non-fat dried milk in PBS containing 0.1% (v/v) Tween 20™ (Sigma) for 60 min at room temperature. Collagens Type I and V were identified using specific antibodies (COL-1 and

V-3C9). COL-1 recognizes native helical collagen Type I (Sigma) while V-3C9 recognizes collagen Type V (Chemicon, Temecula, CA, USA). The primary antibody was diluted in 1% non-fat dried milk in PBS containing 0.1% Tween 20 (COL-1, 1:1000; V-3C9, 1:1000). Membranes were incubated with the primary antibody at 37°C for 120 min. They were then incubated with anti-mouse IgG peroxidase (1: 20,000 in 1% non-fat dry milk in PBS plus 0.1% Tween 20, Sigma) for 120 min at room temperature. Membranes were rinsed 5 times for 10 min with PBS plus 0.1% Tween 20 between each step and immunoreactions identified by chemiluminescence (ECL, Chemicon), according to the manufacturer's instructions. Lastly, the membranes were exposed to X-ray films (Fisher Scientific, Nepean, ON, Canada).

2.3 Microspheres

Plain silicon oxide microspheres (Plain GM, Corpuscular, Mahopac, NY, USA) and commercial 45S5 Bioglass® microspheres (Bioactive GM, MO-SCI, Rolla, MO, USA) were used at 1 mg/mL in the delivery system. Plain GM contained only SiO_2while bioactive GM contained (in weight %) 45% SiO_2, 24.5% Na_2O, 24.5% CaO and 6% P_2O_5. Plain and bioactive GM size distributions were determined by laser diffractometry using a particle size analyser (Mastersizer 2000, Malvern Instruments, Malvern, UK). Both GM supported on a carbon coating were metallized with platinum at 20 mA for 30 s under 9×10^{-2} mbar Ar atmosphere using the splutter coater Emitech K550 (Emitech, Houston, TX, USA). Scanning electron microscope images (S-4000 Hitachi, Mississauga, ON, Canada, at 3.0 kV, 500× magnification) were obtained at a working distance of 8.1 mm for plain GM and 7.8 mm for bioactive GM.

2.4 BMP

rhBMP-2 from Chinese Hamster Ovary (CHO) cells was purchased from R&D Systems (Minneapolis, MN, USA). pBMP-9 (Ac-CGGKVGKACCVPTKLSPISVLYK-NH_2) was synthesized by Celtek Peptides (Nashville, TN, USA) with a final purity of 98%, then dissolved in ultrapure water pH 6.3. The isoelectric point (pI) was determined as previously described by Bjellqvist et al. [33].

2.5 BMP effect

The ALP activity in MC3T3-E1 preosteoblasts that had been incubated for 1 day with or without pBMP-9 (25, 100, 400 ng/mL), or BMP-2 (50 and 100 ng/mL), in serum-free medium was assayed. The incubated cells were washed twice with ice cold PBS and lysed at 4°C in 70 μL 20 mM Tris-HCl, pH 7.4 containing a tablet of complete mini protease inhibitors (Roche Diagnostics, Indianapolis, IN, USA). The cells were lysed, homogenised and the ALP activity was measured using the EnzoLyte™ pNPP Alkaline Phosphatase Assay kit (AnaSpec, San Jose, CA, USA) according to the manufacturer's instructions. The ALP activity was read off a standard curve obtained with ALP concentrations of 0 to 100 ng/mL. Results were normalized to the total protein amounts.

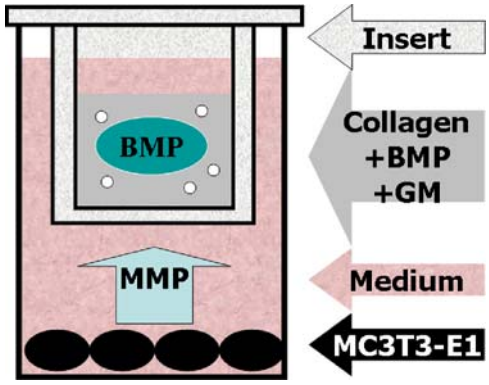

Fig. 1 Diagram of the delivery system

2.6 Delivery system

1 mg/mL GM was included in 1.5 mg/mL collagen diluted in α-MEM. The collagen suspension was adjusted to pH at 7.4 with 18.75% (v/v) 1N NaOH. rhBMP-2 (100 ng/mL) or pBMP-9 (100 ng/mL) was then added to this solution, which was placed in cell culture inserts with porous (0.4 μm) polyethylene terephthalate membranes (Falcon, Fisher Scientific) and left to gel. The delivery system (200 μL) was transferred to 24-well plates containing adherent preosteoblasts cultured in 500 μL α-MEM without FBS (Fig. 1). Then, α-MEM was added both outside (750 μL) and inside (750 μL) the insert. Aliquots (100 μL) of medium were taken from outside the insert on days 1, 3 and 5 after delivery system insertion for the gelatin zymography analysis. Samples were taken for zymography on day 3 and the surrounding medium replaced with α-MEM.

2.7 ELISA assay

The delivery system containing collagen Type I, rhBMP-2 and bioactive GM as described above was transferred to 24-well low adhesion plates (Fisher Scientific). Then, α-MEM was added both outside the insert (1000 μL) and inside it (1000 μL). Aliquots (150 μL) of medium were taken from outside the insert after 7.5, 15, 30 and 60 min and then α-MEM was added outside the insert at each time. The samples were analysed by ELISA assay. Briefly, 96-well ELISA plates (Falcon, Fisher Scientific) were coated overnight with 100 μL antibodies against rhBMP-2 (2 μg/mL , Peprotech, Rocky Hill, NJ, USA) diluted in 0.1M carbonate buffer, pH 9 and then blocked with 0.5% BSA in PBS plus 0.1% Tween

20 (Sigma) for 60 min. Samples (100 μL) were placed in the wells and incubated for 1 h at 37°C. The wells were then washed three times with PBS plus 0.1% Tween 20 and then incubated with 100 μL/well biotinylated antibodies against rhBMP-2 (0.3 μg/mL, Antigenix America, Huntington, NY, USA) for 45 min at 37°C. The washed wells were then filled with 100 μL/well streptavidin-horseradish peroxidase solution (diluted 1/1000 in 0.1% BSA in PBS plus 0.1% Tween 20, Chemicon) and the plate incubated for 30 min. The peroxide colour was developed with *o*-phenylenediamine dihydrochloride substrate (Fast™, Sigma). Then, 100 μL 1N HCl was placed in each well, and the plates were read spectrophotometrically at 492 nm and 540 nm for correction using the microplate reader Synergy™ HT (BioTek, Winnoski, VT, USA). Sample concentrations were calculated from a standard curve generated using rhBMP-2 concentrations from 0 to 100 ng/mL.

2.8 ALP staining

Mouse osteoblast-like cells were stained for ALP on day 5. Medium was removed and each well was washed twice with PBS (pH 7.4). The cells were fixed in 60% (v/v) acetone, 40% (v/v) 0.03M citric acid for 30 s, washed twice with water and stained with Fast Blue RR salt (0.6 mg/mL; Sigma) and Naphthol AS-MX phosphate (0.1 mg/mL; Sigma) for 30 min in the dark. The staining solution was removed and photographs were taken (3 per well) using an Eclipse TE2000-S microscope equipped with a 10X objective and a Retiga 1300R camera (Nikon, Mississauga, ON, Canada).

2.9 Gelatin zymography

The MMP-2 activity of samples was assayed by gelatin zymography. Briefly, 10 μL of the surrounding medium from each well was mixed with NuPAGE LDS 4× sample buffer (Invitrogen) without either thiol reducing agent or heat denaturation. Sample proteins and molecular weight standard (Invitrogen) were separated by SDS-PAGE in 7.5% gel copolymerized with 1 mg/mL gelatin (Sigma) at 50 V for 5 h. Gels were rinsed (2 × 30 min) in 2.5% (v/v) Triton X-100 (Sigma) at room temperature to remove SDS and incubated for 36 h at 37°C in proteolysis buffer (50 mM Tris-HCl pH 7.6, 100 mM NaCl, 5 mM $CaCl_2$ and 0.01% Triton X-100). The gels were then stained with Coomassie blue for 30 min and destained in 40% methanol, 10% acetic acid at least three times for 10 min until white bands appeared clearly from the Coomassie blue background. The destained gels were washed with water (2 × 10 min) and dried in Gel-Dry™ Drying Solution. Scanning images were obtained using FluorChem™ 5500 (Alpha Innotech, San Leandro, CA, USA) and the 62 kDa bands analysed with AlphaEaseFC™ Software, version 4.0.1. (Alpha Innotech). The results from 24 gels were normalized using the Coomassie blue background for each staining series of 4 gels.

2.10 Statistics

All statistical computations were performed with GraphPad Instat® 3.00 software (GraphPad Software Inc., San Diego, CA, USA). The Student Newman Keuls multiple comparison test (ANOVA) or Student's *t*-test were used. Values were considered significantly different if $p < 0.05$.

3 Results

3.1 Characterization of the collagen matrix

The concentration of collagen in the extract was about 3.0 ± 0.2 mg/mL using the Bradford assay and a commercial collagen Type I standard. Both commercial and extracted rat tail tendon collagen showed three bands (about 155, 190 and 210 kDa) when immunoprecipitated with antibodies against collagen Type I (Fig. 2(A)). The bands from the control commercial collagen were less intense than those from the extracted collagen. Dot blot experiments (Fig. 2(B)) showed similar very intense spots specific for collagen Type I and light spots for collagen Type V from both collagen sources. Also, the dots for collagen Type V were more intense in

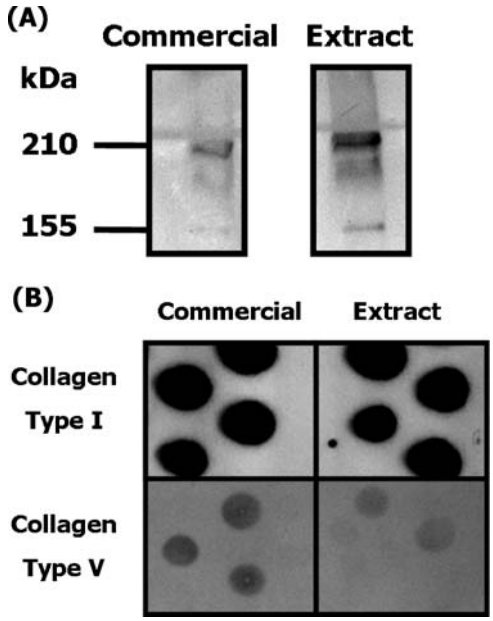

Fig. 2 Proteins (10 μL) in the extracted and commercial collagen were characterised by immunoprecipitation and dot blot. Immunoprecipitation of collagen from both sources with anti-collagen Type I antibody showed three similar bands (A). Dot blot analysis showed more collagen Type V in the commercial collagen than in the extracted collagen (B). Three other independent experiments gave similar results

the commercial collagen than in the extracted rat tail tendon collagen.

3.2 Microspheres

The plain and bioactive GM samples had similar particle size distributions (Fig. 3(A)), with a mean diameter of about 25 μm. The scanning electron microscope images (Fig. 3(B)) confirmed the spherical shapes of the plain and bioactive GM.

3.3 BMP osteogenic potential

We determined the impact of pBMP-9 (25, 100 and 400 ng/mL) and rhBMP-2 (50 and 100 ng/mL) on the differentiation of MC3T3-E1 cells incubated in serum-free medium for 1 day by measuring ALP activity (Fig. 4.), a marker of preosteoblast differentiation [8]. The results are expressed relative to untreated cells (enzyme activity corresponding to 0.17 ± 0.02 ng ALP/μg proteins). The activity of ALP was significantly increased in cells incubated with pBMP-9 or rhBMP-2 over that of untreated cells. The stimulatory effects of pBMP-9 and rhBMP-2 were similar.

3.4 Quantification of released BMP

The ELISA assay developed to measure the BMP-2 release from the delivery system *in vitro* gave a linear absorbance response for BMP-2 concentrations from 0 to 25 ng/mL (R^2 = 99.8%; Fig. 5(A)). The results of the rhBMP-2 release (Fig. 5(B)) revealed that the growth factor diffused slowly (4% after 1 h) and was not influenced by bioactive GM.

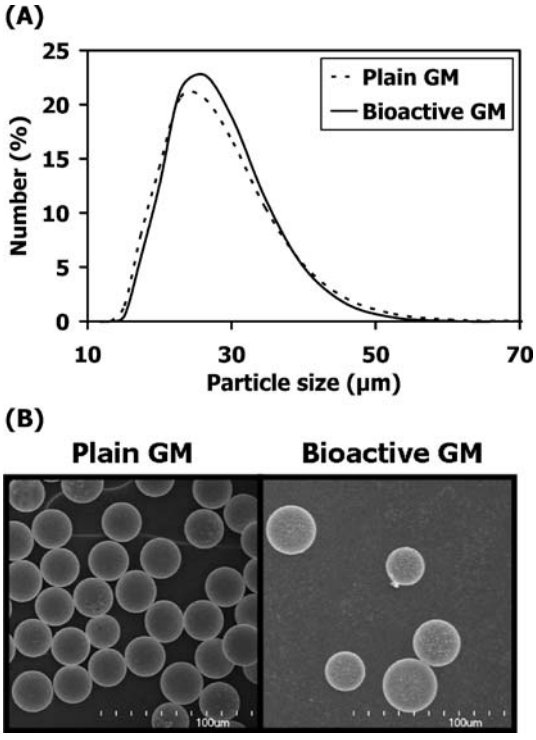

Fig. 3 GM size distributions obtained by light scattering (A) and scanning electron microscopy (B)

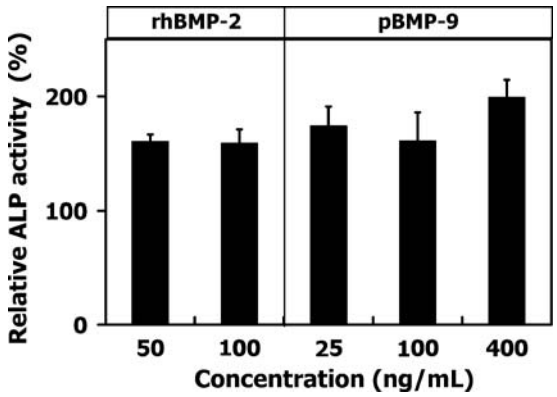

Fig. 4 Relative ALP activity of MC3T3-E1 cells (normalized to the total amount of proteins) as referred to untreated cells on day 1 without the delivery system. Results are means ± SEM of three independent experiments performed in quadruplicate

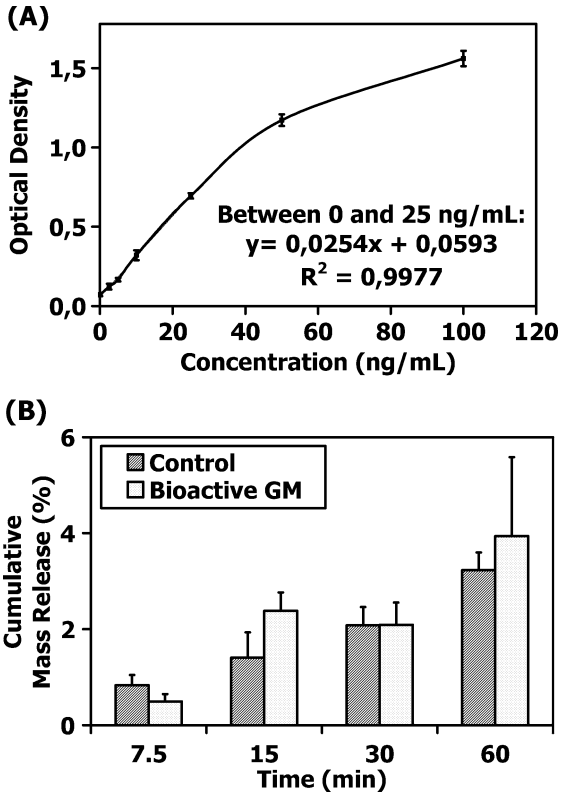

Fig. 5 Quantification of rhBMP-2 by ELISA assay. (A) Standard curve was generated using rhBMP-2 concentrations from 0 to 100 ng/mL. Results are means ± SEM ($n = 3$). Two other independent experiments gave similar results. (B) rhBMP-2 release profile from the delivery system at 7.5, 15, 30 and 60 min. Results are means ± SEM of two independent experiments performed in duplicate

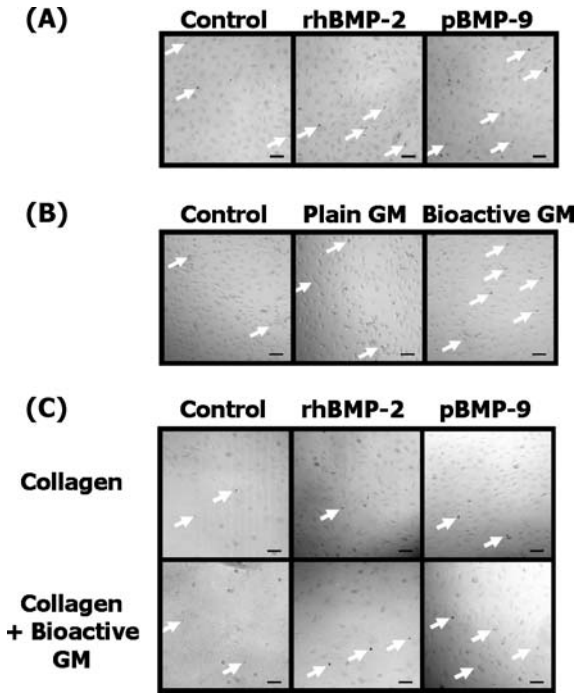

Fig. 6 ALP staining of MC3T3-E1 on day 5 without the delivery system (A) or using the delivery system in the absence (B) or in the presence of BMP (C). Two other independent experiments performed in triplicate gave similar results. The pictures are of representative intra and inter-experiments for each condition. Bar = 100 μm

3.5 Cell differentiation

We analyzed the effect of the delivery system on pre-osteoblast behaviour on day 5. Flow cytometry gave a cell viability of over 90%, fewer than 3.5% apoptotic cells under all experimental conditions. Then, cell differentiation was indicated by staining for ALP which appeared as small dark dots (arrows, Fig. 6(A), (B) and (C)). Direct stimulation with rhBMP-2 (no delivery system; Fig. 6(A)) showed more ALP activity than in the control. pBMP-9 seemed to have even more effect than rhBMP-2. Tests using the delivery system without growth factor (Fig. 6 (B)) indicated that only bioactive GM increased the ALP staining compared to the control. Since the results obtained with plain GM were similar to those obtained with the control, we only compared the effect of rhBMP-2 and pBMP-9 with control or bioactive GM. The ALP staining stimulated by rhBMP-2 and the control using the delivery system without bioactive GM seemed similar (Fig. 6(C)), while incubation with pBMP-9 seemed to produce greater ALP activity. By contrast, rhBMP-2 had a greater impact than did the control in the presence of bioactive GM. Incubation with pBMP-9 seemed to produce the greatest specific staining, as under other conditions (Fig. 6(C)).

3.6 MMP production

We evaluated the activity of the MMP secreted by pre-osteoblasts into the serum-free medium by gelatin zymography to determine the cell response to the delivery system. We obtained bands at about 58, 62 and 72 kDa (Fig. 7(A) and (B)). The major gelatinolytic activity at 62 kDa corresponded to the active form of MMP-2, while the small 72 kDa band was the pro-MMP-2 latent form [27, 34]. The 62 kDa band was analysed densitometrically (Fig. 8(A) and (B)). Since medium was changed on day 3, the intensity of the 62 kDa band increased with time (day 1 and 3) under all experimental conditions, then decreased at day 5.

Without the delivery system (Fig. 8(A)), only pBMP-9 significantly increased MMP-2 compared to the control on days 1 and 3. Both rhBMP- 2 and pBMP-9 seemed to stimulate the production of more intense band than the control

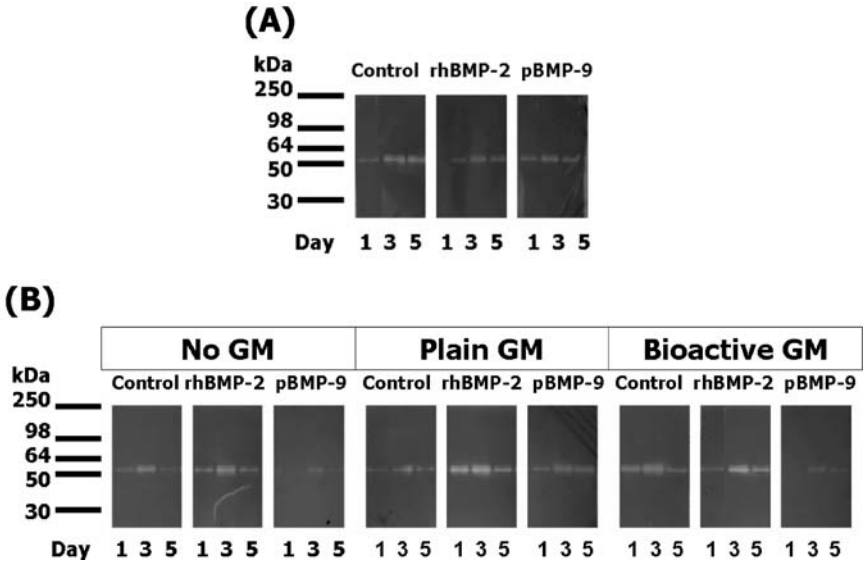

Fig. 7 Zymography gel performed on 10 μL surrounding medium on days 1, 3 and 5 without the delivery system (A) or with the delivery system (B). This experiment was performed in triplicate

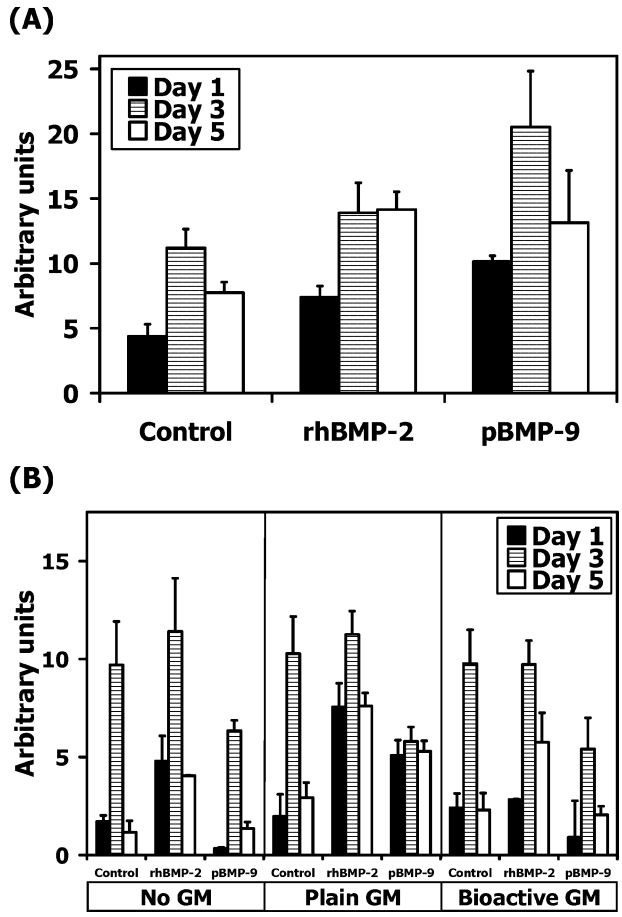

Fig. 8 Densitometric analysis of the 62 kDa band on zymography gel without the delivery system (A) or with the delivery system (B). Results are means ± SEM ($n = 6$)

on day 5. When pBMP-9 was added in the delivery system, with or without GM, it resulted in a significantly less intense 62 kDa band (Fig. 8(B)) than did direct pBMP-9 treatment (Fig. 8(A)) on day 3.

Using the delivery system without GM, rhBMP-2 resulted in significantly more MMP-2 than did pBMP-9 or the control on day 1. With plain GM, only rhBMP-2 produced an effect that was significantly greater than the control on day 1. By contrast, the delivery system with bioactive GM induced similar MMP-2 content in all experimental conditions on day 1. Incubation for 3 days with the delivery system containing either plain or bioactive GM showed that only rhBMP-2 significantly increased the 62 kDa band intensity compared to pBMP-9. On day 5, rhBMP-2 induced a significant raise of MMP-2 compared to the control and pBMP-9, except for pBMP-9 used with plain GM.

4 Discussion

We have shown the influence of a new delivery system using pBMP-9 or rhBMP-2 in collagen Type I gel with bioactive GM on the responses of MC3T3-E1 cells *in vitro*. We first demonstrated that the collagen matrix extracted from rat tail tendons contained mainly collagen Type I. Since collagen Type V can be associated with fibrillar collagen Type I in rat tail tendons [35], dot blots were done to determine its abundance in our collagen. Extracted collagen contained less collagen Type V than did commercial collagen Type I. Furthermore, the extracted collagen had properties that made it a promising carrier for a delivery system, such as a fast gelation (below 30 s) and stability over time until used. Collagen Type I is known to be a biocompatible, biodegradable, malleable and easily handled carrier [12, 36].

Also, we evaluated the rhBMP-2 release in our delivery system using collagen gel and bioactive GM. Indeed, the release profile of rhBMP-2 into a collagen matrix *in vitro* is important for checking the reproducibility of the delivery system and determining the stability of rhBMP-2 [1, 12]. rhBMP-2 possesses two identical 114-residue monomers with a 3D structure about 70 × 35 × 30 Å [37]. The rhBMP-2 in this study was produced by CHO cells and had a molecular weight of about 36kDa and a pI of about 8.5 [3, 12, 38]. Several studies have shown that the retention of rhBMP-2 in the collagen matrix depends on the pH, anionic concentration, cross-linking, reticulation and physical configuration of the collagen [12, 38]. Since the pI of collagen is below pH 7, interactions between rhBMP-2 and collagen are mainly regulated by pH under physiological conditions [12]. Our delivery system using collagen gel and bioactive GM retained 96% of the rhBMP-2 after one hour. Previous studies on the pharmacokinetics of rhBMP-2 in a collagen sponge found that the matrix retained 75% of the rhBMP-2 after 3h, regardless of the collagen source or the rhBMP-2 concentration [39]. Also, collagen carriers produced as a sponge release less rhBMP-2 than collagen suspensions [13].

The bioactive GM also produced a rhBMP-2 release profile similar to that of the collagen control. Since 2.4 kDa pBMP-9 has a pI of about 9, we postulated that our delivery system allowed its release. This was confirmed by the significant stimulation of preosteoblast differentiation and the inhibition of MMP-2 secretion by pBMP-9. Nevertheless, we will characterize the release of pBMP-9 in further experiments using different initial concentrations. The release of both rhBMP-2 and pBMP-9 will also be monitored for a longer time since these bioactive GM can adsorb proteins on their surface [40] which modulate the nucleation and growth state of the apatite layer [41]. Then, we demonstrated the impact of both BMP in our delivery system combining collagen gel and bioactive GM on MC3T3-E1 cell differentiation using ALP staining. BMPs [8, 32, 42, 43] and bioactive GM [21, 23, 24] increase the ALP activity in osteoblastic cells. Incubation with 25 ng/mL pBMP-9 for 1 day is enough to induce significant cell differentiation,

as assessed by ALP activity. Both rhBMP-2 and pBMP-9 (100 ng/mL) had similar effects. Previous studies have shown that incubation for 48h with 10 ng/mL rhBMP-2 significantly increases intracellular ALP activity in MC3T3-E1 cells [32]. We therefore used a dose of 100 ng/mL in all experimental condition to overcome the slow diffusion of growth factors in the delivery system. The effect of BMPs on cell differentiation at longer times was confirmed using ALP staining. Incubation with rhBMP-2 without delivery system for 5 days results in more intense staining for ALP than in controls. pBMP-9 stimulates more cell differentiation than rhBMP-2 at 100 ng/mL. Preliminary quantitative experiments on cells incubated for 7 days revealed that cells incubated directly with pBMP-9 contained 25% more ALP activity than cells incubated with rhBMP-2 (data not shown). The peptide derived from BMP-9 therefore seems to be a good stimulator of preosteoblast differentiation. BMP-9 is one of the most effective inducers of cell differentiation in the BMP subfamily of cytokines, particularly as an inducer of osteoblasts [5, 44].

In the presence of the delivery system, pBMP-9 also induced the greatest preosteoblast differentiation. Surprisingly, the effect of rhBMP-2 was not very different from that of the control. The presence of bioactive GM also increased preosteoblast differentiation under all experimental conditions. This is in accordance with other studies demonstrating the positive effect of bioactive GM on MC3T3-E1 preosteoblast differentiation [21, 22]. Välimäki et al. [45] have shown that recombinant adenovirus carrying human BMP-2 cDNA gene transfer and bioactive GM have a synergetic promoting effect on new bone formation *in vivo*. We find that the optimal delivery system for cell differentiation after incubation for 5 days is a combination of pBMP-9 and bioactive GM in collagen gel.

Preosteoblast differentiation induced by ascorbate-2-phosphate increases production of the active form of MMP-2 [26]. Since both rhBMP-2 and pBMP-9 favor cell differentiation, we assessed the ability of these growth factors to induce MMP secretion by MC3T3-E1 preosteoblasts in culture medium without ascorbate-2-phosphate. Previous studies have demonstrated that these cells secrete both MMP-9 latent form and the active form of MMP-2. The major band produced under our experimental conditions is the 62 kDa active form of MMP-2 [27, 34]. Without the delivery system, pBMP-9 increases the production of this enzyme on day 3 in comparison to rhBMP-2. Also, incubation with either rhBMP-2 or pBMP-9 for 5 days resulted in MMP bands of similar intensity. Interestingly, pBMP-9 produced a less intense MMP-2 band than did rhBMP-2 using the delivery system.

5 Conclusion

We showed that pBMP-9 has a greater effect on the differentiation of MC3T3-E1 cells than does rhBMP-2. When pBMP-9 is used in our delivery system, it stimulates the greatest preosteoblast differentiation and the lowest MMP-2 secretion. Thus, our delivery system based on a collagen Type I gel, pBMP-9 and bioactive GM is a promising system for bone healing. Future studies will optimise this delivery system by determining the influence of pBMP-9 concentration.

Acknowledgments We thank the Material Characterization Centre from Université de Sherbrooke for measuring particle size distributions and Dr Owen Parkes for editing the English text. This research was supported through a Natural Sciences and Engineering Research Council of Canada program.

References

1. V. LUGINBUEHL, L. MEINEL, H. P. MERKLE and B. GANDER, *Eur. J. Pharm. Biopharm.* **58** (2004) 197.
2. M. R. URIST, *Science* **150** (1965) 893.
3. J. M. WOZNEY, V. ROSEN, A. J. CELESTE, L. M. MITSOCK, M. J. WHITTERS, R. W. KRIZ, R. M. HEWICK and E. A. WANG, *Science* **242** (1988) 1528.
4. A. H. REDDI, *Cytokine Growth Factor Rev.* **16** (2005) 249.
5. H. CHENG, W. JIANG, F. M. PHILLIPS, R. C. HAYDON, Y. PENG, L. ZHOU, H. H. LUU, N. AN, B. BREYER, P. VANICHAKARN, J. P. SZATKOWSKI, J. Y. PARK and T.-C. HE, *J. Bone Joint Surg. Am.* **85** (2003) 1544.
6. N. SYKARAS and L. A. OPPERMAN, *J. Oral Sci.* **45** (2003) 57.
7. F. KUGIMIYA, S. OHBA, K. NAKAMURA, H. KAWAGUCHI and U. CHUNG, *J. Bone Miner. Metab.* **24** (2006) 95.
8. Y. TAKUWA, C. OHSE, E. A. WANG, J. M. WOZNEY and K. YAMASHITA, *Biochem. Biophys. Res. Commun.* **174** (1991) 96.
9. M. KANATANI, T. SUGIMOTO, H. KAJI, T. KOBAYASHI, K. NISHIYAMA, M. FUKASE, M. KUMEGAWA and K. CHIHARA, *J. Bone Miner. Res.* **10** (1995) 1681.
10. A. R. POYNTON and J. M. LANE, *Spine* **27** (2002) S40.
11. H. J. SEEHERMAN, M. BOUXSEIN, H. KIM, R. LI, X. J. LI, M. AIOLOVA and J. M. WOZNEY, *J. Bone Joint Surg. Am.* **86** (2004) 1961.
12. M. GEIGER, R. H. LI and W. FRIESS, *Adv. Drug Deliv. Rev.* **55** (2003) 1613.
13. H. SEEHERMAN and J. M. WOZNEY, *Cytokine Growth Factor Rev.* **16** (2005) 329.
14. S. R. WINN, H. ULUDAG and J. O. HOLLINGER, *Adv. Drug Deliv. Rev.* **31** (1998) 303.
15. R. H. LI and J. M. WOZNEY, *Trends Biotechnol.* **19** (2001) 255.
16. H. SEEHERMAN, J. WOZNEY and R. LI, *Spine* **27** (2002) S16.
17. M. KATO, H. TOYODA, T. NAMIKAWA, M. HOSHINO, H. TERAI, S. MIYAMOTO and K. TAKAOKA, *Biomaterials* **27** (2006) 2035.

18. E. P. BARBOZA, A. L. CAULA, F. DE O. CAULA, R. O. DE SOUZA, L. GEOLAS NETO, R. G. SORENSEN, X. J. LI and U. M. E. WIKESJO, *J. Periodontol.* **75** (2004) 702.
19. D. EGLIN, S. MAALHEEM, J. LIVAGE and T. CORADIN, *J. Mater. Sci.: Mater. Med.* **17** (2006) 161.
20. M. M. PEREIRA, A. E. CLARK and L. L. HENCH, *J. Am. Ceram. Soc.* **78** (1995) 2463.
21. S. HATTAR, A. ASSELIN, D. GREENSPAN, M. OBOEUF, A. BERDAL and J.-M. SAUTIER, *Key Eng. Mat.* **254–256** (2004) 749.
22. S. HATTAR, A. ASSELIN, D. GREENSPAN, M. OBOEUF, A. BERDAL and J.-M. SAUTIER, *Biomaterials* **26** (2005) 839.
23. M. BOSETTI, L. ZANARDI, L. HENCH and M. CANNAS, *J. Biomed. Mater. Res.* **64A** (2003) 189.
24. I. D. XYNOS, M. V. J. HUKKANEN, J. J. BATTEN, L. D. BUTTERY, L. L. HENCH and J. M. POLAK, *Calcif. Tissue Int.* **67** (2000) 321.
25. R. ALA-AHO and V.-M. KAHARI, *Biochimie* **87** (2005) 273.
26. A. MIZUTANI, I. SUGIYAMA, E. KUNO, S. MATSUNAGA and N. TSUKAGOSHI, *J. Bone Miner. Res.* **16** (2001) 2043.
27. K.-I. SHIMOKAWA, M. KATAYAMA, Y. MATSUDA, H. TAKAHASHI, I. HARA, H. SATO and S. KANEKO, *Mol. Hum. Reprod.* **8** (2002) 32.
28. M. P. LUTOLF, F. E. WEBER, H. G. SCHMOEKEL, J. C. SCHENSE, T. KOHLER, R. MULLER and J. A. HUBBELL, *Nat. Biotechnol.* **21** (2003) 513.
29. Y. HIRAKI, H. INOUE, C. SHIGENO, Y. SANMA, H. BENTZ, D. M. ROSEN, A. ASADA and F. SUZUKI, *J. Bone Miner. Res.* **6** (1991) 1373.
30. N. R. JORGENSEN, Z. HENRIKSEN, O. H. SORENSEN and R. CIVITELLI, *Steroids* **69** (2004) 219.
31. C. A. LUPPEN, E. SMITH, L. SPEVAK, A. L. BOSKEY and B. FRENKEL, *J. Bone Miner. Res.* **18** (2003) 1186.
32. T. NAKASE, K. TAKAOKA, K. MASUHARA, K. SHIMIZU, H. YOSHIKAWA and T. OCHI, *Bone* **21** (1997) 17.
33. B. BJELLQVIST, B. BASSE, E. OLSEN and J. E. CELIS, *Electrophoresis* **15** (1994) 529.
34. D. ZHOU, H. S. LEE, F. VILLARREAL, A. TENG, E. LU, S. REYNOLDS, C. QIN, J. SMITH and K. L. P. SUNG, *J. Orthop. Res.* **23** (2005) 949.
35. S. RICARD-BLUM and F. RUGGIERO, *Pathol. Biol.* **53** (2005) 430.
36. K. GELSE, E. POSCHL and T. AIGNER, *Adv. Drug Deliv. Rev.* **55** (2003) 1531.
37. C. SCHEUFLER, W. SEBALD and M. HULSMEYER, *J. Mol. Biol.* **287** (1999) 103.
38. W. FRIESS, H. ULUDAG, S. FOSKETT, R. BIRON and C. SARGEANT, *Int. J. Pharm.* **185** (1999) 51.
39. H. ULUDAG, D. D'AUGUSTA, R. PALMER, G. TIMONY and J. WOZNEY, *J. Biomed. Mater. Res.* **46** (999) 193.
40. K. D. LOBEL and L. L. HENCH, *J. Sol-Gel Sci. Technol.* **7** (1996) 69.
41. H. H. LU, S. R. POLLACK and P. DUCHEYNE, *J. Biomed. Mater. Res.* **54** (2001) 454.
42. C. Y. CHUNG, A. IIDA-KLEIN, L. E. WYATT, G. H. RUDKIN, K. ISHIDA, D. T. YAMAGUCHI and T. A. MILLER, *Biochem. Biophys. Res. Commun.* **265** (1999) 246.
43. T. NATSUME, S. TOMITA, S. IEMURA, N. KINTO, A. YAMAGUCHI and N. UENO, *J. Biol. Chem.* **272** (1997) 11535.
44. Q. KANG, M. H. SUN, H. CHENG, Y. PENG, A. G. MONTAG, A. T. DEYRUP, W. JIANG, H. H. LUU, J. LUO, J. P. SZATKOWSKI, P. VANICHAKARN, J. Y. PARK, Y. LI, R. C. HAYDON and T. C. HE, *Gene Ther.* **11** (2004) 1312.
45. V.-V. VALIMAKI, J. J. YRJANS, E. I. VUORIO and H. T. ARO, *Tissue Eng.* **11** (2005) 387.