The Effects of Recombinant Human BMP-7, Prepared from a COS-7 Expression System, on the Proliferation and Differentiation of Rat Newborn Calvarial Osteoblasts

Dong Hee Lee[1], Hwal Suh[1], Dong-Wook Han[1], Bong Joo Park[1], Jin Woo Lee[2], and Jong-Chul Park[1]

Departments of [1]Medical Engineering and [2]Orthopedic Surgery, Yonsei University College of Medicine, Seoul, Korea.

A family of proteins, the bone morphogenetic proteins (BMPs), which promote osteoblast differentiation and bone mineralization, have recently been identified. One, BMP-7, has shown the ability to induce cartilage and bone formation processes. In this report, the possibility that other cell lines, to CHO cells, may also be available as host cells for the expression of hBMP-7 was validated. Recombinant human BMP (rhBMP)-7 was produced in COS-7 cells, as a processed mature disulfide-linked homodimer, with an apparent molecular weight of 36,000. Examination of the expressions of the markers characteristic of osteoblast phenotypes showed that the rhBMP-7 specifically stimulated the inductions of alkaline phosphatase (ALP) (5-fold increase at 100 ng of rhBMP-7/ml), parathyroid hormone (PTH)-mediated intracellular cAMP production (4-fold increase at 100 ng of rhBMP-7/ml) and osteocalcin synthesis (5-fold increase at 100 ng of rhBMP-7/ml). In summary, the in vitro mineralization assay results provide evidence that the rhBMP-7 peptide, produced by COS-7 expression system, possesses intact biological activity. A similar pattern of biological activity was observed for the BMP-7 in COS-7 cells compared to the corresponding CHO cell expression system. Thus, these findings can be experimentally utilized for the production of rhBMPs for *in vitro* or *in vivo* studies.

Key Words: Bone morphogenetic protein, COS-7, rat newborn calvaria cell, osteoblast, DNA synthesis, alkaline phosphatase, cAMP, osteocalcin

INTRODUCTION

Bone morphogenetic protein-7 (BMP-7; also called osteogenic protein-1), a member of the BMP subfamily of the transforming growth factor-β (TGF-β) superfamily, was originally isolated from bone, and shown to induce ectopic bone formation in a rat subcutaneous bone induction model.[1-3] Several members of this family (BMPs 2-15) have been identified, and their corresponding genes cloned from human complementary DNA libraries.[4-11] BMPs are hetero- or homo-dimeric proteins, synthesized as larger precursors, which are processed to produce the biologically active carboxy-terminal domains, the 110 - 140 amino acids. The amino acid sequences of these proteins show a highly conserved 7-cysteine domain at the C-terminal end, which suggests that they are members of the TGF-β superfamily.[12] Recent studies have shown that human BMPs/Ops, generated by recombinant DNA technology, are capable of inducing new cartilage and bone formation *in vivo*, and restoring large bone defects in various animal models.[4,13,14] Through recombinant gene technology, BMPs are available in sufficient amounts for basic research and clinical trials. Of the BMPs tested in heterotopic and orthotopic locations, recombinant human (rh) BMP-2 and rhBMP-7 induced structurally sound orthotopic bone in various experimental systems. rhBMP-7, produced in Chinese hamster ovary (CHO) cells, induces bone formation, *in vivo*, and increases cell proliferation and collagen synthesis of osteoblasts *in vitro*.[13] In addition, rhBMP-7 stimulates the

increased expressions of markers characteristic of osteoblast phenotypes, namely alkaline phosphatase (ALP) activity, osteocalcin synthesis and parathyroid hormone (PTH) stimulated intracellular cAMP levels, in rat calvaria cells.

In this report, the possibility of using other cell lines for the transfection of a variety of different cDNAs was validated, and these may also be available as host cells for the expression of hBMP-7. rhBMP-7 was produced in COS-7 cells, as a processed mature disulfide-linked homodimer, with an apparent molecular weight of 36,000. This report describes the characterization of the purified rhBMP-7, and demonstrates that it is able to promote cell proliferation. In addition, the rhBMP-7 was shown to be effective in specifically stimulating the markers characteristic of osteoblast phenotypes, *e.g.* ALP, osteocalcin, PTH-mediated cAMP.

MATERIALS AND METHODS

Cell cultures

The COS-7 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, NY, USA) with 4.5 g/l glucose, containing 10% fetal bovine serum (FBS; Gibco Laboratories) and antibiotics (100 U/ml of penicillin-G and 100 μg/ml of streptomycin). The rat neonatal calvaria cells (RNC) were isolated from the calvaria of 1-day-old Sprague Dawley rats, by a sequential enzyme digestion, as described previously.[1] Briefly, the calvaria were minced and incubated, at room temperature for 20 min, with gentle shaking of an enzymatic solution, containing 0.1% collagenase, 0.05% trypsin and 4 mmol/l Na_2-ethylenediaminetetraacetic acid (EDTA) in calcium and magnesium-free PBS. This procedure was repeated, yielding a total of six digests. The cells isolated from digests four to six were cultured separately in DMEM (10% FBS). After reaching a subconfluent state, the cells were removed from each flask and combined as the osteoblasts. The cells were used for all experiments in the second passage, as described below.

Preparation of BMP-7

COS-7 cells, secreting rhBMP-7, were established, as previously described,[9] with the exception of COS-7 cells being used, instead of Chinese hamster ovary cells, for the transfection of the BMP-7 cDNA. Briefly, the cDNA molecules encoding the hBMP-7 were synthesized by a polymerase chain reaction method, using the two primers (5′GCTAGGATCCCCTCTGCCACCTGGG GCGGTGC and 5′GCTAGTCGACGCCCCAAAG GGTCTGAATTCTCG), for recognizing the 5′ starting and 3′ ending regions of the cloned BMP-7 cDNA, respectively. The amplified cDNA was inserted into a pUC19 vector. A 1.5-kbp SalI/ BamHI fragment was recovered, and inserted into the pTARGET expression vector (Promega, Madison, USA). The COS-7 cells were transfected with the SuperFect™ transfection reagent (QIAGEN Inc., Valencia, CA, USA), and the stable BMP-7 transformants selected, by their resistance to the drug G418, and subcultured.

The hBMP-7 was purified from conditioned medium using three chromatographic steps: S-Sepharose, phenyl-Sepharose (Pharmacia LKB Biotechnology Inc., Piscataway, NJ, USA) and reverse phase HPLC (Waters Delta Prep 4000 HPLC, equipped with a Bondapark C18, 125 A and 25 × 100 mm cartridge column; Waters Corp., Milford, MA, USA). Two liters of conditioned medium, containing 0.5% FBS, was diluted with 2 volumes of 9 M urea and 20 mM HEPES at pH 7.0, was applied to a column of S-Sepharose, equilibrated with 6 M urea and 20 mM HEPES at pH 7.0, also containing 50 mM NaCl. After washing with the equilibration buffer, step elution of the bound protein was accomplished with the same buffer, containing 100 and 300 mM NaCl. The 300 mM NaCl fraction was made up to a concentration of 1.0 M ammonium sulfate, and applied to a phenyl-Sepharose column, pre-equilibrated with 6 M urea, 1.0 M ammonium sulfate, 0.3 M NaCl and 20 mM HEPES at pH 7.0. After washing with the equilibration buffer, the column was step-eluted with the same buffer, containing 0.6 M ammonium sulfate and water. The protein eluted with water was then sequentially dialyzed against water and 30% acetonitrile and 0.1% trifluoroa-

cetic acid, and finally subjected to C18 reverse phase HPLC, as described previously.[16] The fractions containing hBMP-7, as determined by western blot analysis and silver staining, were pooled. The specific polyclonal anti-human BMP-7 antibody (Peptron Inc., Daejeon, Korea) was produced from a rabbit immunized with purified epitide (epitope sequence of BMP-7, STGSKQRS QNRSKTC), conjugated with KLH, through the sulfhydryl group, by the MBS coupling method. The purity of the hBMP-7 was determined by gel scanning densitometry. The amounts of isolated hBMP-7 were determined by HPLC, based on the absorbance peak of BMP-7 at 214 nm, in reference to known quantities of a purified rhBMP-7 standard (R & D Systems Inc., Minneapolis, MN, USA).

Biological assay of BMP activity

The biological activity of rhBMP-7 was assayed using RNC cells for the cell proliferation and osteogenic differentiation markers: ALP, for the production of cAMP in response to PTH, and osteocalcin. To determine the effects of rhBMP-7 on the osteoblasts' growth, the RNC cells were examined by determining the rate of [^{3}H] thymidine incorporation into the total acid-insoluble DNA. The DNA synthesis rates were determined in triplicate cultures, after 24 h of rhBMP-7 treatment, by adding [methyl-^{3}H] thymidine (2 μCi/ml; NEN Life Science Products, Boston, MA, USA) for 6 h prior to the termination of the culture. The incorporation was terminated by aspiration of the medium, and after washing three times with PBS, the trichloroacetic acid (10%)-precipitated radioactive DNA was extracted with 1% (w/v) sodium dodecyl sulfate (SDS) and 0.1 M NaOH and quantitated using a liquid scintillation counter (1450 MicroBeta Trirux; Wallac, Turku, Finland).

The ALP activity was measured using a commercial assay kit (Sigma, St. Louis, MO, USA). The cells were lysed by sonication, on 0.05% Triton X-100, in PBS, for 60 s at room temperature. The total cellular ALP activity in the lysates was measured in 2-amino-2-methyl-1-propanol buffer at pH 10.3 and 37℃, with p-nitrophenyl phosphate as the substrate. The reactions were

terminated by the addition of 0.5 M NaOH, and the absorbance of the reaction mixture measured at 405 nm, using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The total protein level in the lysates was measured according to the Bradford assay, using bovine serum albumin as the standard. The ALP activity was expressed as nanomoles of p-nitrophenol liberated per microgram of total cellular protein.

To histochemically examine alkaline phosphatase activity, the cells were fixed for 10 min, with 3.7% formaldehyde, at room temperature. After washing with PBS, the cells were incubated for 20 min, with a mixture of 0.1 mg/ml of naphthol AS-MX phosphate (Sigma), 0.5% N,N-dimethylformamide, 2 mM MgCl$_2$ and 0.6 mg/ml of fast blue BB salt (Sigma), in 0.1 M Tris-HCl at pH 8.5 and room temperature.

To determine the production of cAMP in response to PTH, the cells were pre-incubated for 20 min, with a culture medium containing 0.5% BSA and 1 mmol/L 3-isobutyl-1-methylxanthine. The pre-incubation medium was removed, and each well incubated for 8 min, with 200 ng/ml of human PTH (hPTH, Sigma) dissolved in the same culture medium. The cAMP concentration in the cell layer was determined by a radioimmunoassay, using a cAMP assay kit (PerSeptive Biosystems, Framingham, MA, USA).

The osteocalcin concentrations in conditioned medium samples were measured by a radioimmunoassay, using mouse osteocalcin reagents (Biomedical Technologies, Inc., Stoughton, MA, USA). The total DNA content in cell layers was determined by a fluorometric assay, based on the reaction of m-diaminobenzoic acid dihydrochloride (DABA · 2HCl) with free deoxyribose, as previously described.[17] In brief, the DNA concentration in the 5% trichloroacetic acid-precipitated cell layers was determined against a calf thymus DNA standard, using a fluorescence spectrophotometer (Hitachi F-2000, Hitachi Instruments, Inc., San Jose, CA, USA) to measure the fluorescence emission at 500 nm, with excitation at 408 nm. The osteocalcin production was expressed as nanograms of mouse osteocalcin in the conditioned medium per microgram of cell layer DNA.

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RESULTS

The full-length rhBMP-7 cDNA clone, encoding the rhBMP-7 precursor, including the signal sequence, was expressed in mammalian cells (COS-7) in order to obtain the correctly processed and fully active protein. The cell clones expressing the rhBMP-7 were selected on the basis of Western blot analysis, using rhBMP-7 specific antisera. Purification of the rhBMP-7, from the COS-7-conditioned medium, yielded preparations of the processed mature rhBMP-7 with greater than 90% purity. Fig. 1 shows immunoblotted and Coomassie-stained aliquots of the purified preparations of rhBMP-7 following SDS-PAGE. The COS-7 rhBMP-7 migrated as a dimer of approximately 36 kDa, which after reduction migrated

at approximately 18 kDa.

The effect of the rhBMP-7 on the mitogenesis of RNC cells was examined in the absence of serum, using subconfluent cultures, and compared with the mitogenic response elicited by fresh FBS (0.5-10%). The results showed that the rhBMP-7 stimulated the DNA synthesis in subconfluent cultures, as examined by [^{3}H]thymidine incorporation into the total acid-insoluble DNA (Fig. 2). Compared with the control cultures, the rhBMP-7 showed a 2-3 fold maximal mitogenic stimulation, at approximately 100 ng/ml, in the serum-free medium. The mitogenic activity of 100 ng rhBMP-7/ml in the serum-free medium was comparable to that elicited by 10% FBS alone (Fig. 2).

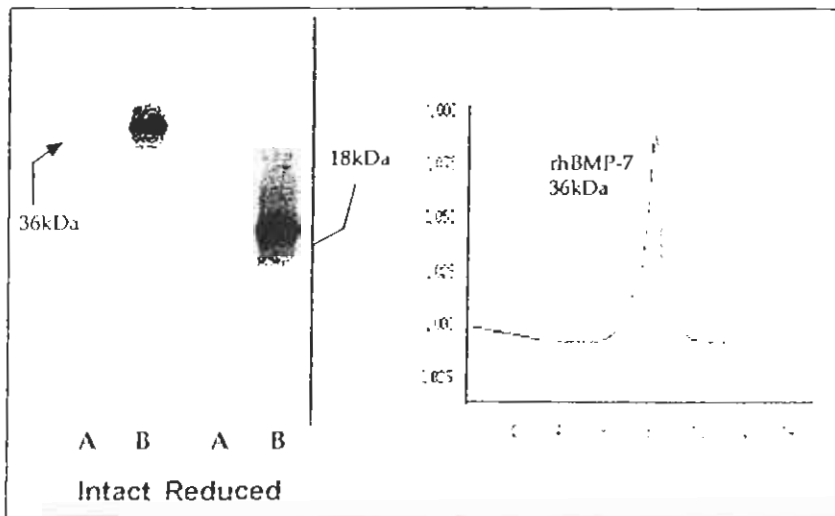The effects of the rhBMP-7 on the osteoblast markers, or the activities known to be associated

Fig. 1. The image on the left is a photograph showing the results of the SDS-PAGE and western blot analyses of the purified COS-7 cell produced rhBMP-7. COS-7 conditioned media were purified, as described in Materials and Methods. A pool of the active fractions from the C18 reverse phase chromatography purified rhBMP-7 was analyzed by SDS-PAGE, without a reducing agent and after reduction with DTT. Lanes designated as A are immunoblots and lanes designated as B are silver-stained. The image on the right is a gel filtration chromatogram (Shodex OHpak SB-803 column) of rhBMP-7
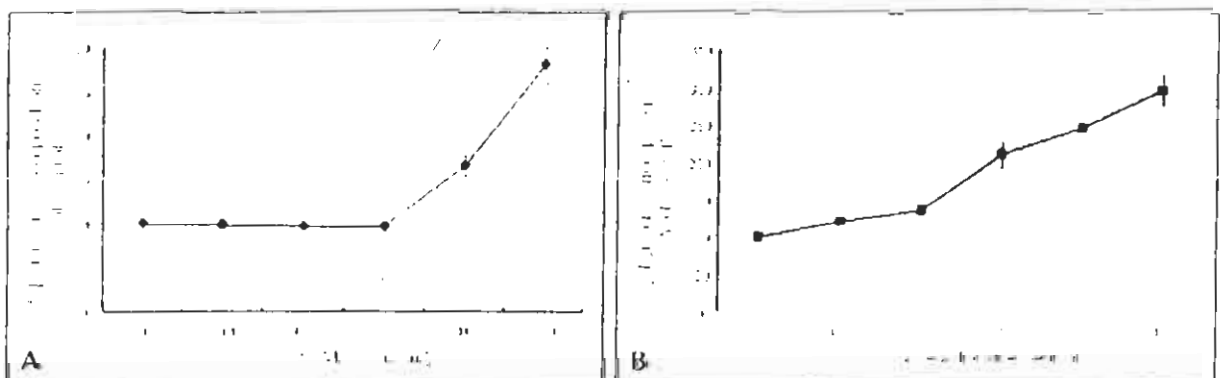

Fig. 2. Effect of rhBMP-7 and FBS on the DNA synthesis in RNC cell cultures. The effect of rhBMP-7 on the mitogenesis of RNC cells was examined in the absence of serum, using subconfluent cultures (A), and compared with the mitogenic response elicited by fresh FBS (B) (0.5-10%). Data represent the means ± SD of five cultures for each treatment

with their cellular functions, were examined using osteoblast cultures. The specific properties examined included: the alkaline phosphatase activity (a marker enzyme for osteogenesis), the PTH-mediated cAMP production (a marker for the hormonal responsiveness of osteoblasts) and the synthesis of osteocalcin (a bone-specific protein, and a marker for mature osteoblast activity and bone formation).

As shown in Fig. 3, the rhBMP-7 stimulates the ALPase specific activity in a dose-dependent manner: by 4- and 5-fold at 50 and 100 ng/ml rhBMP-7, respectively (Fig. 3A). In a kinetic experiment, the ALPase activity of both the rhBMP-7 (100 ng/ml)-treated and control cultures increases gradually, and the activity in the rhBMP-7-treated

culture was consistently higher throughout the culture period (Fig. 3B). In the histochemical analysis of the ALP activity, 100 ng/ml rhBMP-7 produced detectable levels of ALP activity, as revealed by the histochemical staining for ALP (Fig. 4). In the RNC cell cultures, the ALP-positive cells were almost not observed in those cultures without rhBMP-7. In contrast, treatment with 100 ng/ml rhBMP-7 increased the number of ALP-positive cells.

We assessed the PTH response by measuring the production of cAMP in RNC cells treated for 9 days, with or without 100 ng/ml rhBMP-7. rhBMP-7 significantly elevated the PTH responses in the RNC cells, compared to that in the rhBMP-7-untreated cells (Fig. 5). In the RNC cells,
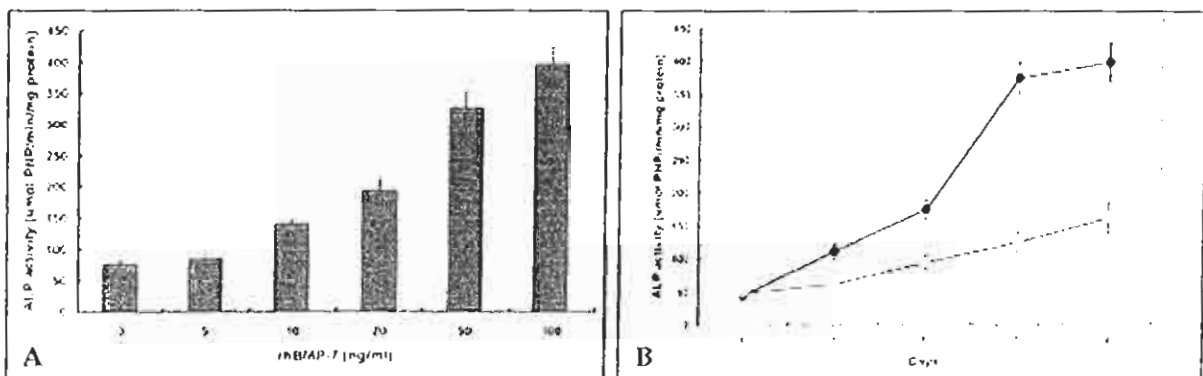

Fig. 3. The effects of rhBMP-7 on the alkaline phosphatase specific activity of the RNC cells, showing the dose dependence (A) and the time course (B) of the changes. For the dose dependence, the cells were cultured with graded concentrations of rhBMP-7 for 9 days, which were harvested on day 9. For the kinetic studies, the cells were cultured without rhBMP-7 (○), or with 100 ng/ml of rhBMP-7 (●), from days 1 to 9, and harvested every 2 days beginning on day 1. Data represent the means ± SD of five cultures for each treatment.
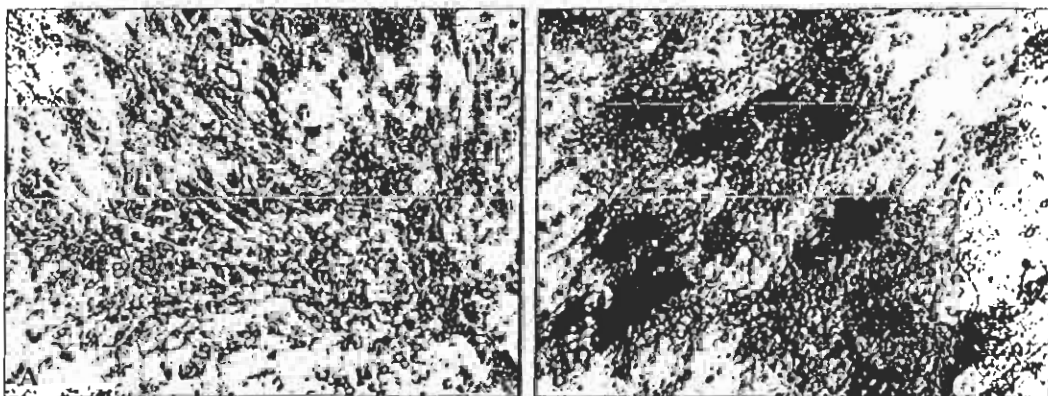

Fig. 4. The effects of rhBMP-7 on the pre-matured osteoblast (RNC cells) differentiation. The RNC cells were cultured for 5 days, with culture medium in the absence (A), or presence (B) of 100 ng/ml rhBMP-7. The cells were stained for ALP activity, as described in Materials and Methods. Blue staining represents ALP activity.

the maximum extent of stimulation of the PTH response, induced by rhBMP-7, was increased 5.3-and 4.6-fold after 7 and 9 days, respectively, compared to the corresponding control cultures without rhBMP-7 (Fig. 5B).

The specific osteoblastic marker, osteocalcin, was also quantitated in the rhBMP-7-treated culture medium. The treatment with rhBMP-7 (100 ng/ml) significantly induced 1,25-(OH)$_2$ vitamine D3-dependent osteocalcin production in the RNC cells (Fig. 6). To confirm the stimulatory effects of rhBMP-7 on the production of osteocalcin, dose-response experiments were conducted using the RNC cells. At concentrations > 20 ng/ml, the rhBMP-7 significantly induced 1,25-(OH)$_2$ vita-

mine D3-dependent osteocalcin production in the RNC cells. In contrast, the non-treated RNC cells had no effect on the osteocalcin production in the presence of 1,25-(OH)$_2$ vitamine D3 after 9 days. In the RNC cells, the maximum extent of stimulation of the osteocalcin production induced by the rhBMP-7 was increased 2- and 5-fold after 5 and 9 days, respectively, compared to the corresponding control cultures without rhBMP-7 (Fig. 6B).

DISCUSSION

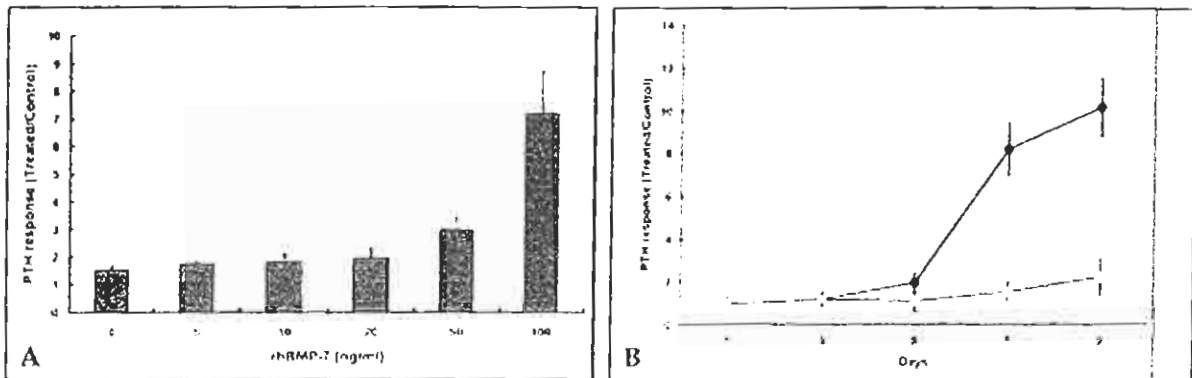In order to produce rhBMP-7, the it's encoding

Fig. 5. Stimulatory effects of rhBMP-7 on the production of cAMP in response to PTH, in pre-matured osteoblasts (RNC cells), in short-term cultures [the dose-dependent effect of rhBMP-7 at 9 days (A), and the time-course effect of the rhBMP-7 (100 ng/ml) (B)]. For the kinetic studies, the cells were cultured without rhBMP-7 (○), or with 100 ng/ml of rhBMP-7 (●), from days 1 to 9, and harvested every 2 days beginning on day 1. Data represent the means ± SD of five cultures for each treatment.
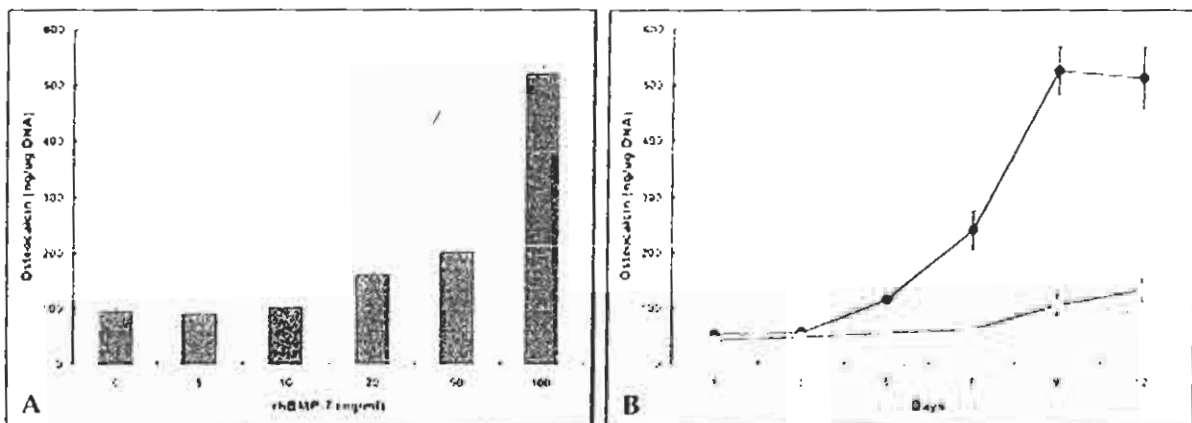

Fig. 6. The effects of rhBMP-7 on the production of osteocalcin by the RNC cells, which shows the dose dependence (A) and the time course (B) of osteocalcin levels in 9-day conditioned medium. The experimental protocol is described in Fig. 3. For the kinetic studies, the cells were cultured without rhBMP-7 (○), or with 100 ng/ml of rhBMP-7 (●), from days 1 to 12, and harvested every 2 days beginning on day 1. Data represent the means ± SD of five cultures for each treatment.

DNA was transferred into the pTARGET expression vector, which was then introduced into COS-7 cells, using conventional genetic engineering techniques. It was contemplated that the preferred expression system for the biologically active recombinant human proteins would be stably transformed mammalian cells. These include, signal and propeptide cleavage, N-linked glycosylation and dimerization. It was further contemplated that the preferred mammalian cells would be CHO cells. Sampath, et al.[17] previously reported that they produced the rhBMP-7 in CHO cells, as a processed mature disulfide-linked homodimer, with an apparent molecular weight of 36,000. The examination of the rhBMP-7 in a rat subcutaneous bone induction model demonstrated that the rhBMP-7 was capable of inducing new bone formation, with a specific activity comparable to that exhibited by highly purified bovine osteogenic protein preparations.

As an alternative expression system, COS-7 cells were employed as the host cells for the BMP-7 expression. This present study describes the purification and characterization of the rhBMP-7 homodimers produced in COS-7 cells. As predicted by analogy to the other members of the TGF-β superfamily, the rhBMP-7 gene was produced as a processed mature disulfide-linked homodimer, as determined by Western blot analysis, with BMP-7 antisera, and the SDS-PAGE analyses, under nonreducing and reducing conditions.

In the cell culture studies, the rhBMP-7 stimulated the osteoblast proliferation, which was dependent on the concentration of the rhBMP-7. The mitogenic response elicited by the rhBMP-7 in serum free medium was comparable to that obtained by the addition of fresh 10% FBS. Natural preparations of osteogenin (BMP-3), from bovine bone,[18,19] and recombinant BMP-2[20-22] have been shown to stimulate the growth of preosteoblasts, periosteal cells and C26 clonal rat osteoblast progenitors, and to inhibit the proliferation of established osteoblast like, MC3T3-E1 and C20 clonal rat osteoblast cells. rhBMP-4, a gene product closely related to BMP-2, has recently been shown to stimulate DNA synthesis in rat osteoblast-enriched cultures.[23] Our results show that rhBMP-7 stimulates the proliferation of preosteo-

blasts in cultures.

The specific activity of ALP, the production of cAMP in response to PTH and osteocalcin synthesis are all enhanced in preosteoblasts cultures in response to rhBMP-7. The effect of rhBMP-7 on ALP induction appears to be specific to osteoblasts, since it was not observed with non-osteoblastic cultures. The rhBMP-7 induced ALP activity remained elevated over the entire period following exposure to rhBMP-7. Recombinant BMP-2, BMP-4 and natural preparations of osteogenin have also been shown to stimulate ALP in calvarial osteoblasts, and MC3T3-E1, periosteal and mouse C3H10T1/2 cells.[18-23] With respect to the rhBMP-7 regulation of other markers of the osteoblast phenotype, the production of cAMP, in response to PTH, was not only maintained by the presence of rhBMP-7, but also increased with increasing amounts of rhBMP-7 in these cultures. The adenylate cyclase activity, which is subject to PTH stimulation, is a biochemical marker of the mature osteoblastic phenotype,[24] and rhBMP-7 increases its activity. Our study showed that rhBMP-7 was also capable of stimulating osteocalcin synthesis in osteoblast-enriched cultures, without the addition of 1,25(OH)$_2$ vitamin D3. Osteocalcin, a bone-specific protein, is known to be synthesized by mature rat and human primary osteoblasts in long term culture.[25,26]

The efficient expression of BMP-7, by recombinant gene technology, has been generally reported in studies on transfected-CHO and vaculovirus infected-insect cells.[16,27,28] Some studies [13,16,29,30] have reported that the treatment of new born rat calvarial cells (or osteoblast-like cells), with rhBMP-7, resulted in a concentration dependent increase in the osteogenic parameters, such as ALP, the adenylate cyclase activity and the production of osteocalcin. However, previous studies have shown that the optimal concentration of rhBMP-7 for the stimulation of osteoblastic differentiation slightly differed (BMP-7 does in the nanogram range) according to the cell type (osteoblast or progenitor osteoblast) and culture conditions used. Therefore, the osteogenic activity of the COS-7 expressed-rhBMP-7 could not be directly compared with that of rhBMP-7 reported in other studies. Although further characterization of the COS-7 system-expressed rhBMP-7 is war-

ranted, a similar pattern of biological activities was observed for the rhBMP-7 produced by the two expression system (CHO and COS-7 expression system, data not shown) in our experimental conditions.

In summary, the results demonstrated that the rhBMP-7 produced by the COS-7 expression system stimulated the osteoblastic differentiation in RNC cultures, and suggest that an important target cell for BMPs was an early osteoprogenitor cells. Taken with the present data, these results suggest that this COS-7 expression system is a useful tool for providing a convenient source of homodimeric BMP-7, with high osteogenic differentiation-inducing activity.

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