

Recombinant Human Bone Morphogenetic Protein-2 Stimulates Osteoblastic Maturation and Inhibits Myogenic Differentiation In Vitro

Akira Yamaguchi,* Takenobu Katagiri,†§ Tohru Ikeda,* John M. Wozney,|| Vicki Rosen,|| Elizabeth A. Wang,|| Arnold J. Kahn,† Tatsuo Suda,‡ and Shusaku Yoshiki*

Departments of *Oral Pathology and ‡Biochemistry, School of Dentistry, Showa University, Tokyo 142; §School of Pharmaceutical Sciences, Kitasato University, Tokyo 108, Japan; ||Genetics Institute Inc., Cambridge, Massachusetts 02140; and †Department of Growth and Development, University of California at San Francisco, San Francisco, California 94143

Abstract. The in vitro effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on osteogenic and myogenic differentiation was examined in two clonal cell lines of rat osteoblast-like cells at different differentiation stages, ROB-C26 (C26) and ROB-C20 (C20). The C26 is a potential osteoblast precursor cell line that is also capable of differentiating into muscle cells and adipocytes; the C20 is a more differentiated osteoblastic cell line. Proliferation was stimulated by rhBMP-2 in C26 cells, but inhibited in C20 cells. rhBMP-2 greatly increased alkaline phosphate (ALP) activity in C26 cells, but not in C20 cells. The steady-state level of ALP mRNA was also increased by rhBMP-2 in C26 cells, but not in C20 cells. Production of 3',5'-cAMP in response to parathyroid hormone (PTH) was dose-dependently enhanced by adding rhBMP-2 in both C26 and C20 cells, though the stimulatory effect was much greater in the former. There was neither basal expression of

osteocalcin mRNA nor its protein synthesis in C26 cells, but they were strikingly induced by rhBMP-2 in the presence of $1\alpha,25$ -dihydroxyvitamin D₃. rhBMP-2 induced no appreciable changes in procollagen mRNA levels of type I and type III in the two cell lines. Differentiation of C26 cells into myotubes was greatly inhibited by adding rhBMP-2. The inhibitory effect of rhBMP-2 on myogenic differentiation was also observed in clonal rat skeletal myoblasts (L6). Like BMP-2, TGF- β 1 inhibited myogenic differentiation. However, unlike BMP-2, TGF- β 1 decreased ALP activity in both C26 and C20 cells. TGF- β 1 induced neither PTH responsiveness nor osteocalcin production in C26 cells, but it increased PTH responsiveness in C20 cells. These results clearly indicate that rhBMP-2 is involved, at least in vitro, not only in inducing differentiation of osteoblast precursor cells into more mature osteoblast-like cells, but also in inhibiting myogenic differentiation.

ECTOPIC bone formation is elicited at intramuscular sites by implantation of bone inducing factors contained in demineralized bone matrix (28, 35, 36). This indicates that cells of the osteoblast lineage have a close relationship with those of the muscular lineage in their ontogeny, and the development of the two cell lineages may be mutually regulated by some factor(s) stored in bone matrix. The components and the action mechanism of these bone inducing factors had long remained obscure. This is mainly due to the difficulties of purification of these factors and the lack of suitable in vitro bioassay systems.

Recently, a number of laboratories have isolated bioactive proteins which induce cartilage and/or bone formation at the sites implanted (1, 13, 30, 39, 40). Human cDNAs for seven different bone morphogenetic proteins (BMPs),¹ BMP-1,

BMP-2 (BMP-2A[41]), BMP-3 (osteogenin [13]), BMP-4 (BMP-2B[41]), BMP-5, BMP-6 (Vgr-1 [14]), and BMP-7 (OP-1[25, 30]) have been cloned (41, 42). The sequences deduced from these cDNAs have indicated that BMP-2 through BMP-7 are members of transforming growth factor- β (TGF- β) superfamily (14, 25, 41, 42). Furthermore, active recombinant human bone morphogenetic protein-2 (rhBMP-2) has been produced, which formed bone tissue in vivo when it was implanted (40). Although several bone-inducing factors were purified from bone matrix, the direct action of the isolated proteins on the osteoblastic cell lineage has not been fully investigated, except for osteogenin (37, 38). Furthermore, the biological action of the factors on nonskeletal tissues has not been extensively explored; since their biological activity has been evaluated only by their in vivo implantation at ectopic sites (1, 13, 30, 39, 40, 41).

To understand more precisely the mechanism of actions of these bone-inducing factors, a suitable in vitro experimental model is required. We recently established and character-

1. *Abbreviations used in this paper:* ALP, alkaline phosphatase; BMP, bone morphogenetic protein; PTH, parathyroid hormone; rh, recombinant human; TGF- β , transforming growth factor- β .

ized five rat clonal osteoblastic cell lines from neonatal rat calvariae (44). These cell lines express various osteoblastic properties, reflecting different maturation stages. Of these, two cell lines, ROB-C20 (C20) and ROB-C26 (C26), exhibit the extremes in maturation stages of osteoblasts. The C20 expresses a wide spectrum of osteoblastic properties, indicating that it is a mature osteoblastic cell line (44). In contrast, the C26 has characteristics of an osteoblast progenitor cell line, retaining the potential to differentiate into both myotubes and adipocytes (44). But neither of these cells produced osteocalcin (bone Gla protein, BGP), a bone matrix protein specifically produced by osteoblasts (11). C20 and C26 cells thus may be useful tools for determining the role of BMPs in osteogenic and myogenic differentiation.

Here we show that the two cell lines, C20 and C26, are responsive to rhBMP-2: this recombinant cytokine not only stimulated proliferation and differentiation of C26 cells, but inhibited myogenic differentiation of C26 cells in vitro. We also demonstrate that rhBMP-2 strikingly induces the synthesis of osteocalcin in the presence of $1\alpha, 25$ -dihydroxyvitamin D_3 in C26 cells.

Materials and Methods

Recombinant Human Bone Morphogenetic Protein-2 and Transforming Growth Factor- β 1

Recombinant human bone morphogenetic protein-2 (rhBMP-2) was produced by CHO (Chinese hamster ovary) cells and purified as described previously (40). The recombinant protein used in the present study was 80-90% pure as judged by silver-stained bands on SDS gels and also by NH_2 -terminal sequencing. The *in vivo* ectopic bone formation assay revealed that this material was active at 1 μ g, the lowest dose level tested 7 d after implantation.

TGF- β 1 purified from human platelets was purchased from R & D Systems, Inc. (Minneapolis, MN).

Cell Culture

Two clonal rat osteoblastic cell lines, ROB-C20 (C20) and ROB-C26 (C26), were isolated from newborn rat calvaria (44). The two cell lines were plated into 24-multiwell plates (Falcon Labware, Lincoln Park, NJ) at a seeding density of 7×10^3 cells per well and cultured with α -MEM (Gibco Laboratories, Grand Island, NY) containing 10% FBS (HyClone Laboratories, Logan, UT) and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin). To determine the role of rhBMP-2 in the myogenic differentiation, we also used a clonal rat cell line of skeletal muscle myoblasts (L6 cells) (43) obtained from the Japanese Cancer Research Resources Bank (Tsukuba Science City, Japan). L6 cells were maintained in DMEM (Gibco Laboratories) supplemented with 10% FBS and antibiotics. L6 cells were plated in 48-well tissue culture plates (Costar Corp., Cambridge, MA) at a cell density of 1×10^4 cells per well. In some experiments, L6 cells were cultured with DMEM containing 2% FBS to induce differentiation of myoblasts (33).

Growth Experiments

After C26 and C20 cells were cultured for 3 d with various concentrations of rhBMP-2, the cells were detached from the culture dishes by incubating with a trypsin/EDTA solution (0.05% trypsin; 0.02% EDTA), and the cell number was counted using a hemocytometer.

Alkaline Phosphatase Activity

Alkaline phosphatase (ALP) activity was determined by an established technique using *p*-nitrophenyl-phosphate as a substrate (26). Protein concentration was determined using a BCA protein assay reagent (Pierce Chemical Co., Rockford, IL).

3',5'-cAMP Production in Response to Parathyroid Hormone

To determine 3',5'-cAMP production in response to parathyroid hormone (PTH), cells were preincubated for 20 min with α -MEM containing 0.5% BSA and 1 mM 3-isobutyl-1-methylxanthine. After preincubation media were removed, cells were incubated for 8 min with 200 ng/ml of human PTH (hPTH(1-34)) (provided by Dr. Hori, Toyo Jozo Co., Shizuoka, Japan) dissolved in the same culture media. The cAMP concentration in the cell layers was determined by RIA using a cAMP assay kit (Yamasa Co., Chiba, Japan).

Myogenic Differentiation

Myotubes appearing in the C26 cells were detected by immunoreactivity to desmin, a muscle-specific intermediate filament. To detect desmin, cells were fixed for 10 min with a cold acetone/ethanol mixture (50:50), and stained for desmin by an indirect immunoperoxidase technique using a mouse anti-cow desmin mAb (Labsystems, Helsinki, Finland). The desmin-positive cells were visualized using a biotinylated anti-mouse IgG antibody (Vector Laboratories, Burlingame, CA). Myotubes appearing in the L6 cells were identified by morphology according to the method described previously (43).

Northern Blot Hybridization of ALP and Collagens

Total RNA was isolated by the guanidine thiocyanate-caesium chloride method (16). 20 μ g of the isolated RNA were electrophoresed in a 1.2% agarose-formaldehyde gel, and blotted onto Hybond-N membranes (Amersham International, Amersham, UK). The membranes were hybridized with ^{32}P -labeled cDNA probes at 65°C in a rapid hybridization buffer (Amersham International) and washed in 0.1 \times SSC buffer containing 0.1% SDS at the same temperature. cDNA probes used were those for rat ALP (23), rat procollagen α 1(I) (6), and α 1(III) (12), and human β -tubulin.

Measurement of Osteocalcin Synthesis

The C26 cells were treated for 3, 6, or 9 d with or without 1 μ g/ml of rhBMP-2. The culture was incubated for the last 24 h of each culture period with or without 2×10^{-8} M $1\alpha, 25(OH)_2D_3$. Northern blot analysis of osteocalcin was performed as described above using a rat osteocalcin cDNA probe (3). The amount of osteocalcin secreted into the culture media was determined by RIA using a rat osteocalcin assay kit (Biomedical Technologies Inc., Stoughton, MA).

Results

Cell Proliferation

Fig. 1 shows the effect of rhBMP-2 on the growth of C26 and C20 cells. The cells were cultured with graded concentrations of rhBMP-2, then counted on day 3. At dose levels >10 ng/ml, rhBMP-2 increased the growth of the C26 cells, but it inhibited the proliferation of the C20 cells dose dependently (Fig. 1).

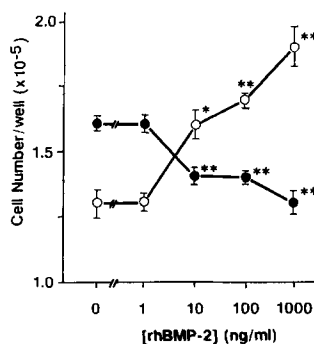


Figure 1. Dose-response effects of rhBMP-2 on the growth of C26 (○) and C20 (●) cells. Cells were plated in 24-well plates and cultured for 3 d with graded concentrations of rhBMP-2. The cells were counted as described in Materials and Methods. Data are means \pm SD of three wells. Significantly different from the control without rhBMP-2. (**p* < 0.05; ***p* < 0.01.)

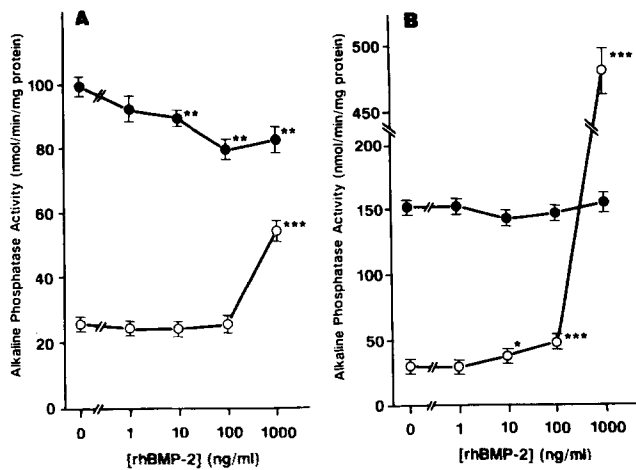


Figure 2. Dose-response effects of rhBMP-2 on ALP activity in C26 (○) and C20 (●) cells. Cells were cultured with graded concentrations of rhBMP-2 for 3 (A) and 6 d (B). ALP activity was determined by the method described in Materials and Methods. Data are means \pm SD of three wells. Significantly different from the control without rhBMP-2. (* p < 0.05; ** p < 0.01; *** p < 0.001.)

ALP Activity

C20 cells exhibited basal activity of ALP approximately five times higher than C26 cells in the absence of rhBMP-2. rhBMP-2 stimulated ALP activity in C26 cells but not in C20 cells. After C26 cells were treated for 3 d with 1 μ g/ml of rhBMP-2, ALP activity was increased to approximately twice the basal level (Fig. 2 A). On day 6, rhBMP-2 increased ALP activity at concentrations <1 μ g/ml. The enzyme activity attained a level 16 times higher than the control level when 1 μ g/ml of the peptide was added (Fig. 2 B). In C20 cells, in contrast, treatment with >10 ng/ml of rhBMP-2, slightly decreased the ALP activity on day 3 (Fig. 2 A). Treatment of C20 cells with rhBMP-2 for 6 d did not induce

any significant changes in the enzyme activity at any concentrations tested (Fig. 2 B).

L6 cells showed a low but detectable ALP activity after they were cultured for 7 d (24.0 ± 1.8 nmol/min per mg protein). Treatment of L6 cells with rhBMP-2 for 6 d increased the enzyme activity to 29.3 ± 0.4 at 10 ng/ml and 32.2 ± 1.9 at 100 ng/ml, but decreased it to 12.5 ± 0.7 nmol/min per mg protein at 1 μ g/ml.

PTH-dependent cAMP Production by C26 and C20 Cells

Both C26 and C20 cells produced cAMP in response to 200 ng/ml of hPTH(1-34), but in the absence of rhBMP-2 the fold induction was higher in C20 cells than C26 cells on both days 3 and 6 (Table I). Treatment with rhBMP-2 dose-dependently increased cAMP production in the two osteoblastic cells, but the C26 cells appeared more sensitive to rhBMP-2 than the C20 cells. On day 6, the maximal increase by 1 μ g/ml of rhBMP-2 was 151-fold in the C26 cells and 58-fold in C20 cells. In L6 cells, there was no increase in the cAMP production in response to PTH in the presence or absence of rhBMP-2.

mRNA Expression of ALP and Collagens

Northern blot analysis showed that rhBMP-2 increased the steady state level of the expression of ALP mRNA approximately twofold in C26 cells, but did not increase it in C20 cells (Fig. 3). There was no appreciable change in the expression of type I and type III procollagens and β -tubulin mRNAs in the C20 and C26 cells treated with rhBMP-2 (Fig. 3).

Osteocalcin Synthesis

Control C26 cells that were cultured for 3-9 d without rhBMP-2 synthesized neither detectable levels of osteocalcin mRNA nor protein even in the presence of 10^{-8} M $1\alpha,25$ -(OH) $_2$ D $_3$ (Fig. 4, A and B). Treatment of C26 cells with 1 μ g/ml of rhBMP-2 for 3-9 d greatly increased the expres-

Table I. Effects of rhBMP-2 on the cAMP Production Stimulated by PTH in C20 and C26 Cells

Cell line	rhBMP-2 ng/ml	Amounts of cAMP produced					
		Day 3			Day 6		
		PTH(-)	PTH(+)	PTH(+)/PTH(-) ratio	PTH(-)	PTH(+)	PTH(+)/PTH(-) ratio
		<i>pmol/well</i>					
C26	0	1.3 \pm 0.0	2.5 \pm 0.0	1.9	0.8 \pm 0.0	12.2 \pm 0.1	15.0
	1	1.6 \pm 0.1	3.1 \pm 0.2	2.0	0.8 \pm 0.0	10.1 \pm 0.1	13.0
	10	1.5 \pm 0.0	4.2 \pm 0.4	2.8	0.8 \pm 0.0	14.3 \pm 0.1	17.9
	100	1.7 \pm 0.0	20.0 \pm 1.5	11.5	1.0 \pm 0.0	60.4 \pm 2.4	62.5
	1,000	2.5 \pm 0.1	110.0 \pm 7.6	48.2	2.5 \pm 0.1	375.0 \pm 9.8	151.4
C20	0	1.4 \pm 0.2	4.2 \pm 0.5	3.0	2.4 \pm 0.1	62.4 \pm 0.8	27.9
	1	1.1 \pm 0.1	4.8 \pm 0.4	4.1	2.0 \pm 0.1	48.0 \pm 2.0	24.7
	10	1.5 \pm 0.1	6.3 \pm 0.2	4.2	2.5 \pm 0.2	66.9 \pm 1.3	26.7
	100	1.4 \pm 0.1	14.7 \pm 1.7	10.7	2.1 \pm 0.1	72.7 \pm 1.6	35.3
	1,000	1.8 \pm 0.3	22.0 \pm 1.2	12.5	1.6 \pm 0.1	92.5 \pm 4.1	58.3

Cells were cultured for 3 or 6 d with graded concentrations of rhBMP-2. On days 3 and 6, cells were treated for 8 min with 200 ng/ml of hPTH(1-34) and the amount of cAMP produced was determined. Data are means \pm SEM of three wells.

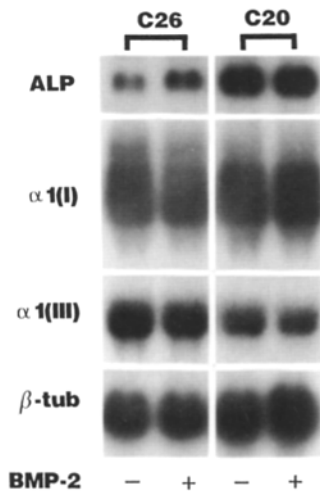


Figure 3. Northern blot analysis of the mRNA expression of ALP and procollagens of $\alpha 1(I)$ and $\alpha 1(III)$. Total RNAs were isolated from C26 and C20 cells after treatment for 3 d with 1 $\mu\text{g/ml}$ of rhBMP-2. 20 μg of total RNA were electrophoresed in agarose gel and hybridized with the respective ^{32}P -labeled cDNA probes as described in Materials and Methods. β -tub, β -tubulin.

sion of osteocalcin mRNA (Fig. 4 B). On day 3, a low level of osteocalcin mRNA was expressed only in the $1\alpha,25(\text{OH})_2\text{D}_3$ -treated cells. On days 6–9, the levels of mRNA increased even in the absence of $1\alpha,25(\text{OH})_2\text{D}_3$, and its expression was strikingly increased by the treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ for 24 h (Fig. 4 B). The amounts of osteocalcin secreted into the culture media were closely related to the mRNA levels of osteocalcin (Fig. 4, A and B).

Myogenic Differentiation

When C26 cells became confluent, some of them spontane-

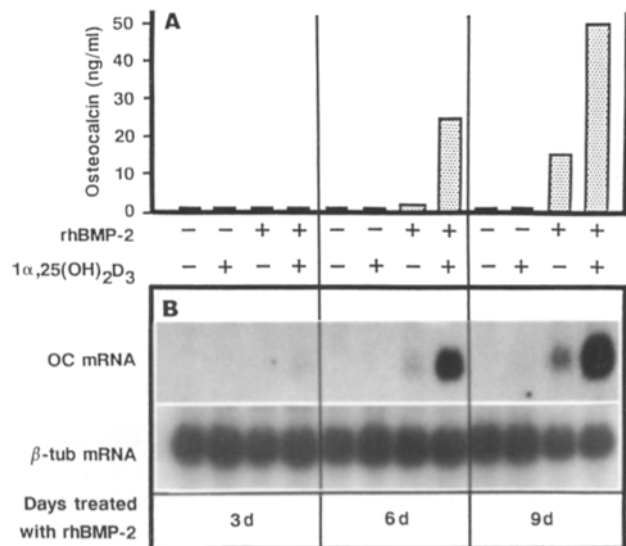


Figure 4. Induction of osteocalcin synthesis by rhBMP-2 in C26 cells. C26 cells (7.9×10^4 cells) were inoculated in 60-mm² culture dishes. After preculturing the cells for 24 h, rhBMP-2 (1 $\mu\text{g/ml}$) was added and the cells were further cultured for 3, 6, or 9 d. The dish was treated for the last 24 h of each culture period with or without 2×10^{-8} M $1\alpha,25(\text{OH})_2\text{D}_3$. (A) The amount of osteocalcin secreted into culture media as determined by RIA using the method described in Materials and Methods. (B) mRNA expression for osteocalcin. 20 μg of total RNA isolated from each sample were electrophoresed in agarose gel and hybridized with a ^{32}P -labeled cDNA probe for osteocalcin as described in Materials and Methods. OC, osteocalcin, β -tub, β -tubulin.

ously differentiated into desmin-positive myotubes (Fig. 5 A). Adding rhBMP-2 dose-dependently decreased the number of desmin-positive myotubes at dose levels >1 ng/ml of the peptide (Figs. 5 B and 6 A). Numerous myotubes also appeared when L6 cells were cultured with the inducing culture media (Fig. 5 C). Treatment of L6 cells with rhBMP-2 at higher concentrations than 1 ng/ml similarly reduced the number of myotubes dose dependently (Figs. 5 D and 6 B).

Effects of TGF- $\beta 1$ on Osteoblastic and Myogenic Differentiation

BMP-2 is a member of the TGF- β superfamily (41). We examined the effect of TGF- $\beta 1$ on osteoblastic and myogenic differentiation to determine whether the effects shown in this study are specific to BMP-2. Like BMP-2, TGF- $\beta 1$ dose-dependently decreased the number of myotubes appearing in C26 cells (Fig. 7 C). However, unlike BMP-2, treatment of C20 cells with 0.1–10 ng/ml of TGF- $\beta 1$ for 6 d decreased ALP activity dose dependently (Fig. 7 A). TGF- $\beta 1$ also suppressed ALP activity in C26 cells, though the inhibitory effect was smaller than in C20 cells (Fig. 7 A). TGF- $\beta 1$ showed no significant effect on the PTH-dependent cAMP production in C26 cells at any concentrations tested (Fig. 7 B), but it increased PTH responsiveness in C20 cells (Fig. 7 B). Treatment of C26 cells for 6 d with 0.1–10 ng/ml of TGF- $\beta 1$ induced no osteocalcin production even in the presence of $1\alpha,25(\text{OH})_2\text{D}_3$.

Discussion

The present study clearly demonstrates that the recombinant BMP-2 poly-peptide affects proliferation and differentiation of certain osteoblastic cells in vitro. To determine if rhBMP-2 had differential effects on osteoblasts at varying stages of differentiation, we used two clonal osteoblast-like cell lines at different stages of differentiation: C26 cells are osteoprogenitor cells, also retaining potentials to differentiate into muscle cells and adipocytes; C20 cells are more differentiated osteoblast-like cells (44). rhBMP-2 stimulated the growth of C26 cells but slightly inhibited the proliferation of C20 cells. The stimulatory effect of rhBMP-2 on the osteoblastic phenotype was much greater in C26 cells than in C20 cells. This indicates that rhBMP-2 preferentially stimulates proliferation and differentiation of osteoprogenitor cells.

At present, osteocalcin is the only known bone-specific protein produced by osteoblasts (11). This protein is reported to appear in a later stage of osteoblast differentiation (2, 34, 45), presumably in mature osteoblasts, whereas ALP (2, 34, 45), PTH receptors (29), and type I collagen (31, 34, 45) appear even in less differentiated osteoblasts. Although C26 cells have already acquired a low level of the latter characteristics, but they lack osteocalcin synthesis in an unstimulated state. It is significant that rhBMP-2 induced osteocalcin mRNA expression and its protein synthesis in C26 cells. Apparently rhBMP-2 induced differentiation of immature osteoblastic cells into mature osteoblasts not only by increasing the expression of the constitutive phenotype present in progenitor cells, but also by inducing the expression of novel genes present only in mature osteoblasts. rhBMP-2 is the first cytokine that is capable of inducing osteocalcin mRNA expression and its protein synthesis. Whether other bone

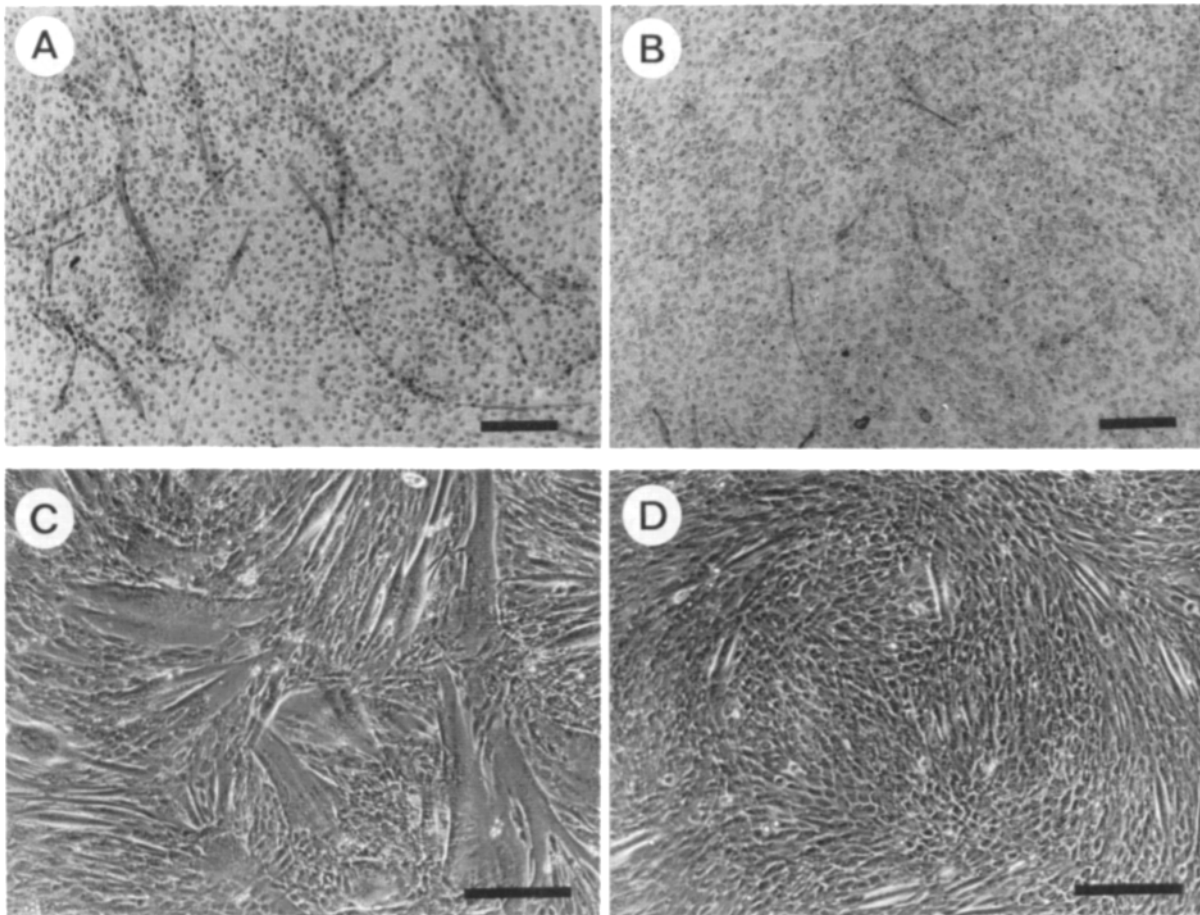


Figure 5. Effects of rhBMP-2 on the development of myotubes in C26 and L6 cells. (A and B) C26 cells were immunostained with an anti-desmin antibody as described in Materials and Methods. Numerous desmin-positive elongated myotubes are seen in the C26 cells cultured for 7 d in the absence of rhBMP-2 (A). Note that there is a marked decrease in the number of desmin-positive myotubes in the C26 cells cultured with rhBMP-2 (100 ng/ml) for 6 d (B). (C and D) Phase-contrast features of L6 cells. The cells were cultured with the media containing 10% FBS for the first 3 d then with the media containing 2% FBS for the last 3 d. Numerous multinucleated myotubes are seen in the L6 cells cultured in the absence of rhBMP-2 (C). Treatment with rhBMP-2 (100 ng/ml) for the last 3 d greatly inhibited the appearance of myotubes (D). Bars, 200 μ m.

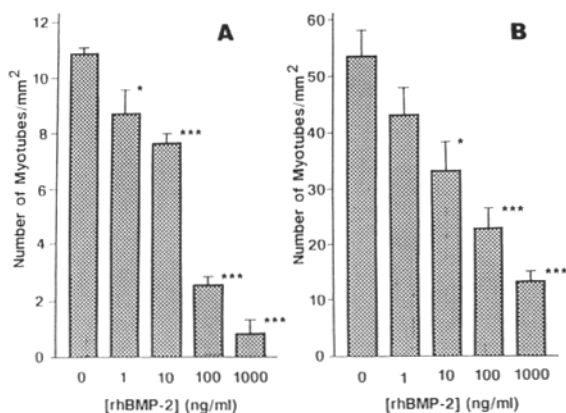


Figure 6. Dose-response effects of rhBMP-2 on the development of myotubes in C26 cells (A) and L6 cells (B). Myotubes appearing in each cell line were identified as described in Materials and Methods. The number of myotubes was counted in 6.25-mm² at the central region of each well. Data are means \pm SD of three wells. Significantly different from the control without rhBMP-2. (* p < 0.05; *** p < 0.001.)

growth factors have similar effects in inducing osteocalcin synthesis remains to be elucidated in the future.

Cells of the osteoblastic phenotype make a significant quality of type I collagen. In the present study, treatment with rhBMP-2 induced no appreciable change of expression of type I and type III procollagen mRNAs in C26 and C20 cells. Collagen synthesis measured by [³H]proline incorporation was not significantly stimulated by rhBMP-2 (100 ng/ml) in C26 cells and in calvarial cultures (unpublished data). No stimulation occurred in the mRNA expression for type I procollagen in the mouse clonal embryonic fibroblastic cells (C3H10T1/2) (unpublished data), which responded to rhBMP-2 in increasing their ALP activity and PTH responsiveness (9). It thus appears that BMP-2 has no appreciable effect on collagen synthesis, at least in the cell lines we used.

The minimal doses of rhBMP-2 that affected proliferation and differentiation of C26 cells were much higher than those of other cytokines and growth factors. Also, the effective doses of rhBMP-2 necessary to induce ALP activity were >10 ng/ml in C3H10T1/2 cells (9) and mouse osteoblast-like

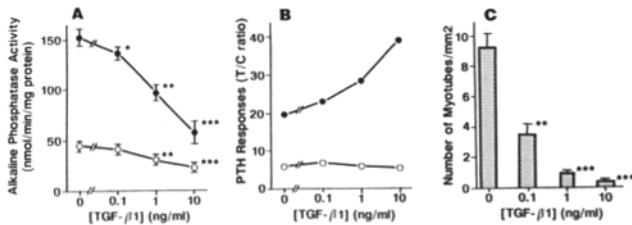


Figure 7. Effects of TGF- β 1 on osteoblastic and myogenic differentiation. (A) Dose-response effects of TGF- β 1 on ALP activity in C26 (○) and C20 (●) cells. After the cells were cultured with graded concentrations of TGF- β 1 for 6 d, the ALP activity was determined by the method described in Materials and Methods. Data are means \pm SD of three wells. (B) Dose-response effects of TGF- β 1 on the cAMP production stimulated by PTH in C26 (○) and C20 (●) cells. The cells were cultured for 6 d with graded concentrations of TGF- β 1. Then the amount of cAMP produced was determined as described in Materials and Methods, after the cells were treated for 8 min with or without 200 ng/ml of hPTH(1-34). The PTH response was expressed as the ratio of cAMP production by PTH-stimulated cells to that by unstimulated cells. Data are means of three wells in each group. (C) Dose-response effects of TGF- β 1 on myogenic differentiation in C26 cells. Myotubes were identified as described in Materials and Methods. The number of desmin-positive cells was counted in 6.25-mm² at the central region of each well. Data are means \pm SD of three wells. Significantly different from the control without TGF- β 1. (* p < 0.05; ** p < 0.01; *** p < 0.001.)

MC3T3-E1 cells (10) (unpublished data). However, this low responsiveness might not be due to the low specific activity of BMP-2. This may be explained by the difference between the native BMP-2 and the recombinant one, since it requires more rhBMP-2 than the native BMPs to induce similar ectopic bone formation (40). Alternatively, it may be due to the sticky nature of BMP-2. Further comparative studies using native and recombinant BMPs are needed to clarify this point.

During the process of ectopic bone formation after bone-inducing factors are implanted into muscular tissues, these factors appear to alter the differentiation of muscle cells around the sites applied. The present study clearly demonstrates that rhBMP-2 inhibits myogenic differentiation in vitro. Apparently BMP-2 has opposite actions on osteogenic and myogenic differentiation in pluripotent cells: rhBMP-2 stimulates differentiation of mesenchymal cells into osteoblastic cells and inhibits differentiation of those cells into muscle cells. It is reported that cartilage formation precedes the osteoblast differentiation in the process of ectopic bone formation induced by BMP-2 (27, 28). Since myogenic cells are induced to differentiate into chondrogenic cells in response to bone matrix in vitro (18, 19, 24, 32), it will be interesting to determine whether rhBMP-2 induces differentiation of C26 cells and L6 cells into chondrogenic cells. This possibility is currently under investigation.

BMP-2 is a member of the TGF- β superfamily (41). To determine whether the effects shown in this study are specific to BMPs, we compared the effects of rhBMP-2 and TGF- β 1 on osteogenic and myogenic differentiation in C26 and C20 cells. Like BMP-2, TGF- β 1 suppressed myogenic differentiation in C26 cells. However, unlike BMP-2, TGF- β 1 decreased

ALP activity in both C26 and C20 cells. TGF- β 1 stimulated PTH responsiveness in C20 cells, but it induced neither PTH responsiveness nor osteocalcin production in C26 cells. In vivo studies (8, 22) have indicated that TGF- β injected into periosteal regions induced new bone formation in rats, but several in vitro experiments have revealed that the effects of TGF- β on ALP activity and PTH responsiveness varied among the cells tested (4, 7, 20, 21). The inconsistency of the in vitro data suggests that the action of TGF- β on osteoblast differentiation depends on the stage of differentiation of the osteoblast-like cells used and the culture conditions. The inhibitory effects of TGF- β on myogenic differentiation have also been demonstrated in other myogenic cells (5, 17). Together, these results indicate that both BMP-2 and TGF- β similarly inhibit myogenic differentiation, but they have different actions in osteoblast differentiation.

Although the precise role of the respective BMPs and their mutual interaction in osteoblast differentiation have to be elucidated in the future, it is interesting that in the recent finding reported by Lyons et al. (15), BMP-2, Vgr-1 (BMP-6), TGF- β 1 and TGF- β 2 mRNAs are expressed in different populations of mesenchymal cells in the developing skeletal system. They also proposed the importance of the coordinated expression of several members of the TGF- β superfamily for the control of progression of specific cell types through the differentiation pathways (15). It is important to determine the specific actin and the mutual interaction of various BMPs and TGF- β s to understand the precise regulatory mechanisms of osteoblast differentiation.

In conclusion, rhBMP-2 is a bone induction factor that induces differentiation of osteoblast progenitor cells into mature osteoblast with the ability to synthesize osteocalcin. Also, rhBMP-2 inhibits myogenic differentiation. So it appears that BMP-2 is involved in both in vivo osteoblastic and myogenic differentiation at the site of implantation. Whether BMP-2 affects other cell differentiation events including chondrogenesis and adipogenesis is of considerable interest and is under investigation in our laboratories.

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