Journal of Cellular Biochemistry 73:11–19 (1999)

ARTICLES

Effect of $1,25(OH)_2D_3$ on Bone Morphogenetic Protein-3 mRNA Expression

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Abstract Bone morphogenetic proteins (BMPs) are members to the transforming growth factor-β superfamily. They induce ectopic bone formation in rat and are pleiotropic initiators of inducible osteogenic precursor cells. A lot of reports have studied the presence of BMPs and their effects on bone marker expression in many different cell lines, however none describe the regulation of BMP3 by different factors and expression conditions. When a human bone marrow stromal cell (HBMSC) culture was treated simultaneously with $1,25(OH)_2D_3$ (10^{-8} M) and BMP3 (2.5 ng/ml), the total osteocalcin content in the cell layer and in the culture medium was higher than when the culture was treated with either factor alone (162%). To elucidate this synergistic activity, Northern blot analysis was done to study the effect of $1,25(OH)_2D_3$ on BMP3 mRNA expression. Several human cell lines (MNNG, U-2OS, MG-63, KHOS, TE85, HOS) and HBMSC were treated by $1,25(OH)_2D_3$ (10^{-8} M for 24 h). Purified mRNA from treated and untreated cells were denatured using glyoxal and dimethylsulfoxide, and were fractionated on a 1% agarose gel. After electrophoresis, RNA were blotted onto a nylon membrane and incubated with ^{32}P-labeled BMP3 and GAPDH riboprobes. Northern blot analysis revealed that, the BMP3 mRNA level was increased in a few cell lines (MG-63, HBMSC, HOS) after the addition of $1,25(OH)_2D_3$ when compared to the untreated cells (127% ± 1; 130.5% ± 19.5; 207% ± 14). An higher stimulation was observed in HBMSC primary culture when compared to differentiated HBMSC. In view of these results, we now investigate the following hypothesis: does the BMP3 promoter exhibit the vitamin D receptor response like the osteocalcin gene? J. Cell. Biochem. 73:11–19, 1999. © 1999 Wiley-Liss, Inc.

Key words: BMP3, $1,25(OH)_2D_3$, regulation; transcription; human bone cells

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGF-β) superfamily [Wozney, 1993; Wozney et al., 1988]. They induce ectopic bone formation in vivo [Wang et al., 1990], and are pleiotropic initiators of inducible osteogenic precursor cells [Vukicevic et al., 1989; Reddi, 1992]. BMPs are separable into different functional groups by their high degrees of homology within the C-terminal domains (seven cysteines) and one group contains only BMP3 because of the low similarity with the other BMPs. Moreover, BMP3 is one of the BMPs closer to members of TGF-β superfamily. In addition to the specific and unique characteristics of BMP3 (osteogenin), we are particularly interested in the role of this protein in osteogenesis [Sampath et al., 1987; Luyten et al., 1989]. This osteoinductive protein stimulates alkaline phosphatase in rat calvarial osteoblasts in vitro [Vukicevic et al., 1989] and rat bone marrow stromal cells [Reddi, 1992]. Recently, BMP3 has been shown to stimulate differentiation of osteoprogenitors in human bone marrow [Amédée et al., 1994]. Mechanisms of action are partially known and the type I and II receptors of a few BMPs have been identified [ten Dijke et al., 1994]. However, the molecular regulation of BMPs, and particularly of BMP3, by different factors and the interaction with orthotropic agents are limited [Faucheux et al., 1997]. Cell culture provides a simple approach to study 1) the effect of growth factors on cell proliferation, mRNA expression and/or protein synthesis, and 2) synergistic or antagonistic action between several factors in combination. For example, BMP2-induced osteocalcin level is strikingly increased after $1,25(OH)_2D_3$ treatment [Yamaguchi et al., 1991]. Concerning TGF-β1, it can inhibit alkaline phosphatase levels induced by BMP2 in

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Received 18 February 1998; Accepted 10 September 1998

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W-20–17 cells or BMP4-stimulated alkaline phosphatase in mk90 cells [Wozney, 1993]. In MG-63 cells, TGFβ inhibited $1,25(OH)_2D_3$-induced osteocalcin production, whereas both factors were additive for alkaline phophatase activity, fibronectin, and collagen type I production [Bonewald et al., 1992]. On the contrary, TGF-β1 antagonizes the $1,25(OH)_2D_3$ effects [Staal et al., 1996]. As previously described by Amédée et al. [1994], when human bone marrow stromal cell cultures were simultaneously treated with both $1,25(OH)_2D_3$ (10^{-8} M) and BMP3 (2.5 ng/ml), the total osteocalcin (OC) content, a bone specific protein, in the culture medium was higher than from cells cultured with either factor alone.

In the present study, we set out to examine the following hypothesis: does $1,25(OH)_2D_3$ affect BMP3 mRNA expression? Six human osteoblast cell lines and differentiated human bone marrow stromal cells (HBMSC) were cultured in the presence or absence of $1,25(OH)_2D_3$ (10^{-8} M for 24 h). The regulation of BMP3 mRNA expression was studied by Northern blot analysis. We observed a stimulating effect of $1,25(OH)_2D_3$ on BMP3 expression in a number of human osteoblastic cell lines and HBMSC. The effect was tested in the presence of cycloheximide and actinomycin D and this indicated that $1,25(OH)_2D_3$ did not affect mRNA stability. It seemed this regulation is not related with the osteocalcin level. However this stimulation is detected at higher level in primary culture without dexamethasone treatment and proliferating cells.

MATERIALS AND METHODS

Materials

Alpha minimum essential medium (α-MEM) and fetal calf serum (FCS) were purchased from Sigma (St. Louis, MO) and Gibco BRL (Grand Island, NY). $1,25(OH)_2D_3$ and [α-^{32} P]-UTP were from Leo Pharmaceuticals (Denmark) and Amersham international (England). Cycloheximide and Actinomycine D were purchased from Sigma. Human BMP3 cDNA was a gift from G.R. Mundy (San Antonio TX). All the human cell lines were a gift from J.T. Triffitt (Oxford, U.K.).

Cell Culture

Human osteoblast cell lines (MNNG, U-2OS, MG-63, KHOS, TE85, HOS) were cultivated at 37°C in a humidified atmosphere of 5% CO_2 in air in a-MEM supplemented with 10% (v/v) fetal calf serum (FCS). Human bone marrow was obtained by iliac aspiration from patients aged 20–60 years and cell culture was performed as described previously [Vilamitjana et al., 1993] with modifications. In brief, cells were separated to give a single cell suspension by sequential passage through syringes fitted with a 16-, 18-, and 21-gauge needle. Cells were plated into 75 cm^2 flasks (Nunc) at 5×10^7 cells/75 cm^2. They were cultured in the same conditions as described above, in addition of dexamethasone (10^{-8} M) and medium changed every 4 days in presence of dexamethasone at the same concentration. Confluence was obtained after 10 days. These cells are called differentiated human bone marrow stromal cells (differentiated HBMSC) and expressed the following bone-marker proteins: osteocalcin (OC), alkaline phosphatase (ALP), type I collagen, osteonectin (ON), and calcium binding protein (CaBP28K) [Faucheux et al., 1998].

$1,25(OH)_2D_3$ Treatment

Human cells (HBMSC and the different cell lines) were seeded into 75 cm^2 flasks. Plating densities differed according to the cell culture and were about $5–20 \times 10^3$ cells/cm^2. Confluent or semi-confluent cultures were then incubated for 24 h in α-MEM serum-free medium followed by incubation for 24 h with $1,25(OH)_2D_3$ at 10^{-8} M. Controls were cultivated in vehicle (ethanol, $<0.05\%$) alone.

Northern Blot Analysis

Total RNA (from treated and untreated cells) was isolated using the guanidinium thiocyanate procedure. Two mg of Poly$^+$ RNA, purified by oligo(dT) magnetic Dynabeads (Dynal UK Ltd., Merseyside, UK) from 22 mg of total RNA, were denatured using glyoxal and dimethylsulfoxide, and were fractionated on a 1% agarose gel. After electrophoresis, RNA was blotted onto a Biodyne nylon membrane (Pall Biosupport, Portsmouth, UK) and incubated with [^{32}P]-labeled BMP3 or glyceraldehyde-3-phosphate deshydrogenase (GAPDH) riboprobes. After prehybridization for 2 h, hybridization was performed overnight at 45°C (or 65°C for GAPDH riboprobe) in a hybridization buffer containing 50% deionized formamide, $5 \times$ SSPE, 1% SDS, 100 mg/ml sonicated, and denatured herring sperm DNA, 0.5% low-fat dried milk. Mem-

branes were washed at appropriate stringency's (2 × SSC-0.1% SDS, 0.1 × SSC-0.1% SDS) and exposed to X-ray film (X-MAT, Kodak) using two intensifying screens at −80°C. Densitometric analysis was performed using OPTIMAS 5.2 image analysis software (DataCell Ltd., Maidenhead, Berks, England). BMP3 expression was normalized to the GAPDH mRNA level and results are expressed in percentage of BMP3 mRNA stimulation compared to untreated cells (mean ± SD).

RESULTS

At confluency, six human osteoblast confluent cell lines (MNNG, U2 Os, MG-63, KHOS, TE85, HOS) and differentiated HBMSC were treated with $1,25(OH)_2D_3$ at 10^{-8} M for 24 h. Northern blot analysis was used to determine a possible effect of $1,25(OH)_2D_3$ on BMP3 expression. Only one mRNA species of 6 kb was found and all cell lines and differentiated HBMSC expressed BMP3 mRNA but at different levels (Fig. 1A). GAPDH expression (1.4 kb) was used as a control of expression. Membranes were exposed at different times to prevent over exposition and autoradiographies were quantified (Fig. 1B) by densitometry at different expositions.

KHOS and MNNG cell lines highly expressed mRNA BMP3. Using densitometric analysis, $1,25(OH)_2D_3$ did not significantly stimulate mRNA expression in KHOS cell line (130% ± 19.5) and had no effect in MNNG cell line as U2 Os and TE-85 cell lines. On the contrary, BMP3 mRNA expression was increased in MG-63 (127% ± 1) and particularly in HOS (207% ± 14) cell lines. This effect was weakly observed in differentiated HBMSC (115% ± 5). To detect a connection between BMP3 and osteocalcin expression levels (OC), the same membranes were hybridized with the human OC cDNA probe (Fig. 1A). Only after $1,25(OH)_2D_3$ treatment, OC mRNA was highly expressed in the MG-63 cell line and differentiated HBMSC, and weakly expressed in KHOS and TE-85 cell lines. MNNG, U-2OS and HOS cell lines did not show the presence of OC mRNA, even after $1,25(OH)_2D_3$ treatment.

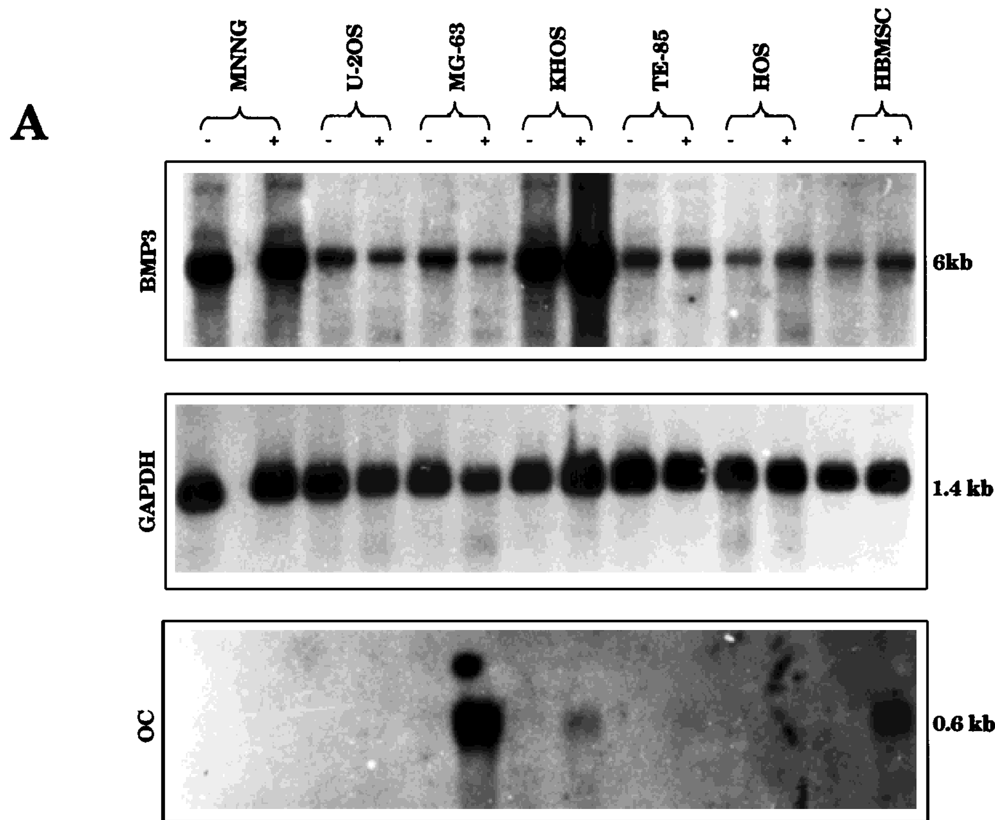
A similar experiment was performed using semi-confluent MG-63 and HOS cell lines (about 80% of confluency). These cells were treated with $1,25(OH)_2D_3$ as previously described and mRNA were used to perform Northern blot analysis (Fig. 2A). Results showed $1,25(OH)_2D_3$-stimulated BMP3 expression is higher in semi-confluent cells (Fig. 2B; 270 % in MG-63 and 230% in HOS cell lines) than in confluent cells.

To determine if this stimulation was only produced by an increase of mRNA stability, we treated semi-confluent cells with cycloheximide (CHX) and actinomycin D (ACT), respectively inhibitors of protein synthesis and translation. Treatment were performed as follow: semi-confluent cells were cultivated in IMDM alone for 24 h. Six hours before $1,25(OH)_2D_3$ treatment, cells were incubated with ACT (10 mg/ml) or CHX (10 or 20 mg/ml). The addition of $1,25(OH)_2D_3$ (10^{-8} M) for 24 h was performed in presence of ACT or CHX at same concentrations. BMP3 mRNA level was quantified by densitometry, GAPDH expression was used as a control of expression (Fig. 3). In the presence of ACT, BMP3 mRNA and GAPDH expression were abolished (data not shown). The CHX addition (Fig. 3) decreased significantly expression level of BMP3 after $1,25(OH)_2D_3$ treatment in MG-63 and HOS cell lines whatever concentration is (10 or 20 mg/ml). This experiment indicated $1,25(OH)_2D_3$ effect is dependent on de novo protein synthesis.

Interaction between $1,25(OH)_2D_3$ and BMP3 has been studied in primary HBMSC cultured without dexamethasone treatment. Bone marrow cell suspension has been cultivated only in IMDM medium supplemented with 10% of FCS for 15 days. Semi-confluent cells were treated with $1,25(OH)_2D_3$ at 10^{-8} M for 24 h and Northern blot analysis was performed from total RNA as previously described in Materials and Methods. Results showed primary HBMSC (without dexamethasone treatment) expressed a weaker level of BMP3 mRNA when compared to differentiated HBMSC cultured in the presence of dexamethasone. However, this expression was highly stimulated with $1,25(OH)_2D_3$ addition (160%) in primary HBMSC (Fig. 4A). Quantification was also performed by densitometry analysis, using GAPDH as control of expression (Fig. 4B).

DISCUSSION

Although purified from bone matrix, BMP3 is produced by many different tissues including brain, lung, kidney, and intestine [Vukicevic et al., 1994] and this agent may control morphogenesis and/or function in these organs. However, a significant role for BMP3 has been shown in cartilage and bone [Hauschka et al., 1988;



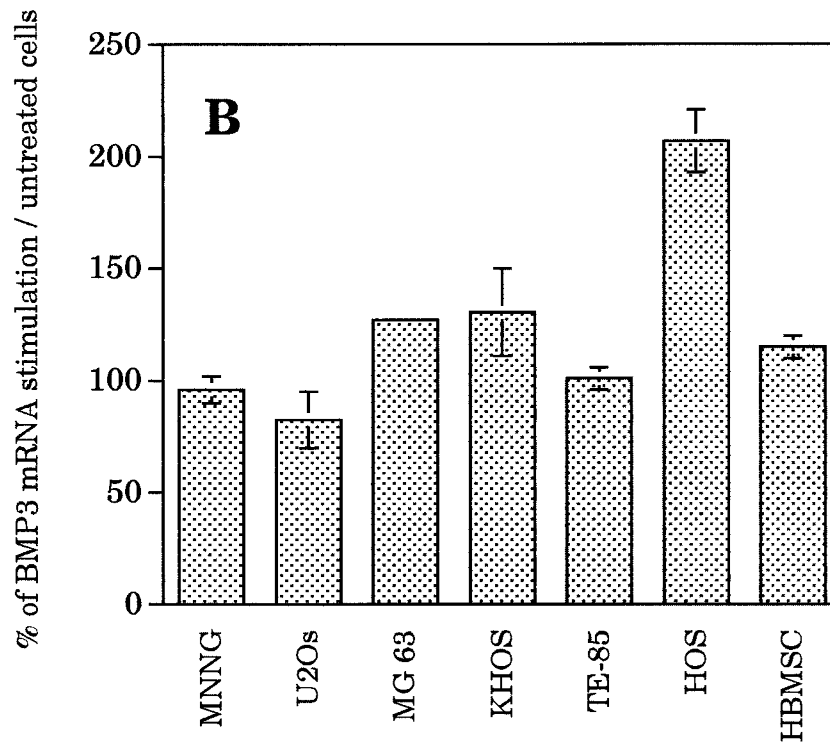
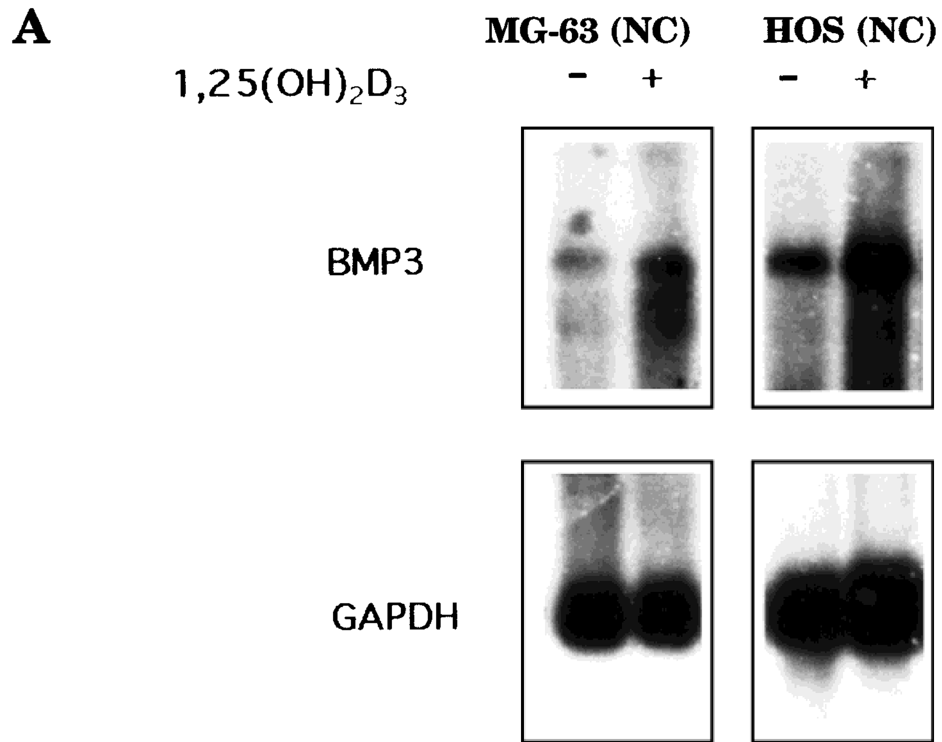


Fig. 1. Effect of $1,25(OH)_2D_3$ on BMP3 mRNA expression in confluent cells by Northern blot. Confluent cell lines (MNNG, U2 0s, MG-63, KHOS, TE-85, HOS) and differentiated HBMSC are incubated in presence (+) or in absence (−) of $1,25(OH)_2D_3$ as previously described. Poly(A) RNA are purified from 22 mg of total RNA and deposited on 1% agarose gel to perform Northern blot (Materials and Methods). The membranes were hybridized with human BMP3 or GAPDH riboprobes and human OC cDNA probe. After washing at different stringencies, the membranes were autoradiographied (**A**) using intensifying screens at −80°C. mRNA size is indicated on the right. Signal intensity is quantified by OPTIMAS 5.2 programme as described in Materials and Methods and BMP3 expression is normalized to the GAPDH mRNA level (**B**; n = 3). Data represent the mean of percentage of stimulation ± standard deviation (SD) when compared to each untreated cells.



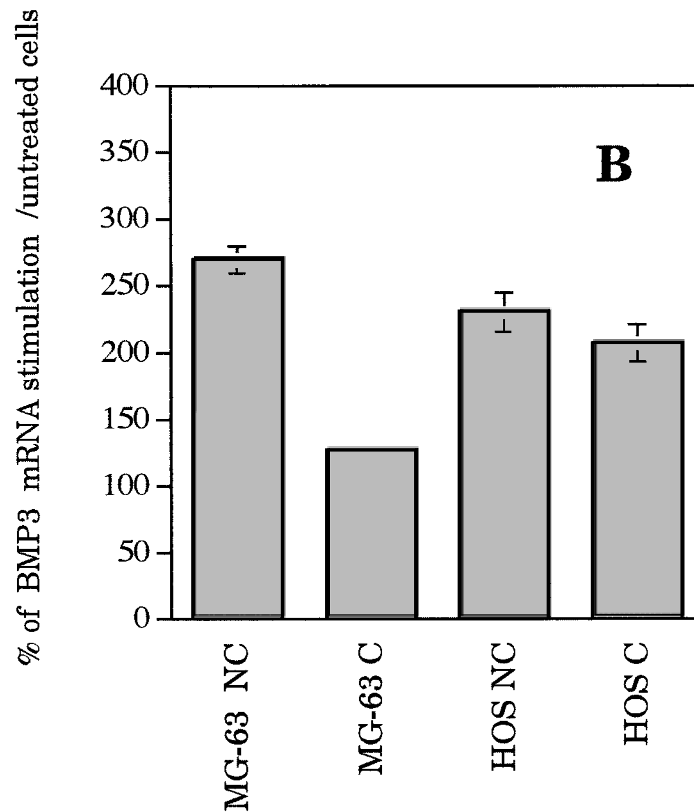


Fig. 2. Effect of $1,25(OH)_2D_3$ on BMP3 mRNA expression in semi-confluent cells by Northern blot. Non-confluent (NC) cell lines (MG-63, HOS) are incubated in presence (+) or in absence (−) of $1,25(OH)_2D_3$ as previously described. After 24 h of treatment, reaction is stopped by washing with 0.1 M PBS pH7.4 and total RNA are extracted as described in Materials and Methods. **A**: Autoradiography of the blot. **B**: Signal intensity is quantified by OPTIMAS 5.2 programme as described in Materials and Methods. BMP3 expression is normalized to the GAPDH mRNA level (n = 3). Data represent the mean of percentage of stimulation ± standard deviation (SD) when compared to each untreated cells. Percentage of BMP3 mRNA stimulation obtained in Figure 1 on confluent cells (C) was also reported in (B).

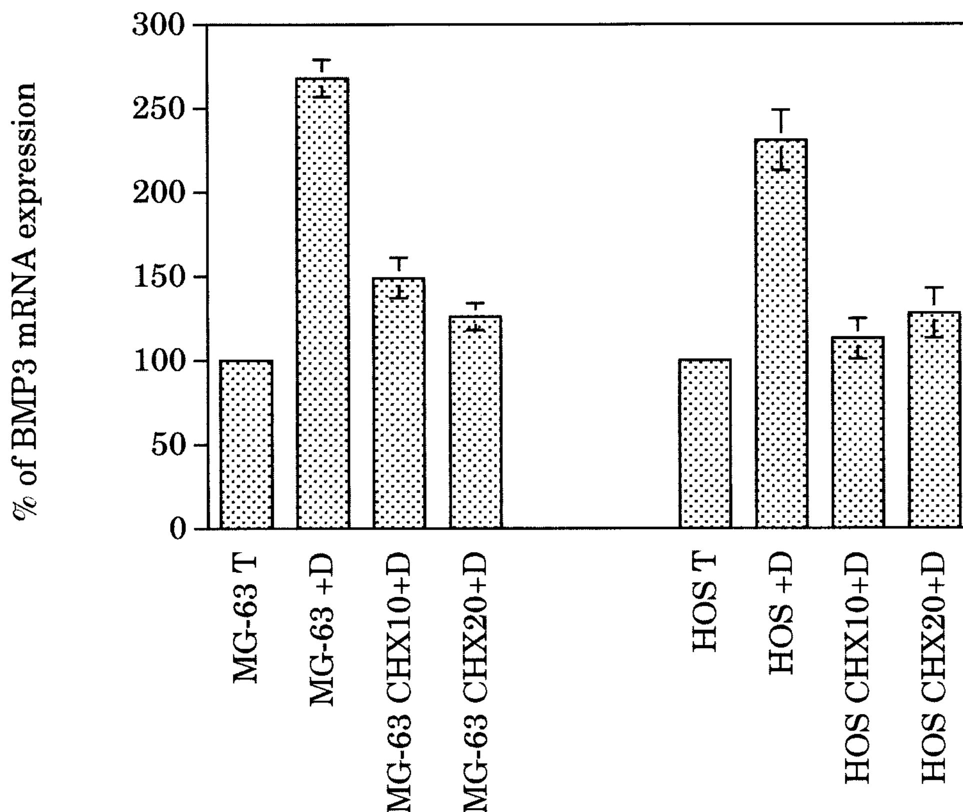


Fig. 3. Cycloheximide (CHX) influency on BMP3 mRNA expression stimulated by $1,25(OH)_2D_3$. Treatment with cycloheximide (CHX) in semi-confluent cells was done as follow: at semi-confluent, two cell lines (MG-63, HOS) are incubated with IMDM medium alone for 24 h, treated by CHX at 10 or 20 mg/ml for 6 h and then by $1,25(OH)_2D_3$ (10^{-8} M; D) for 24 h in presence of CHX. After 24 h of treatment, reaction is stopped by washing with 0.1 M PBS pH 7.4 and total RNA are extracted as described in Materials and Methods. Northern blot was performed as previously described and signal intensity is quantified by OPTIMAS 5.2 programme. BMP3 expression is normalized to the GAPDH mRNA level (n = 2). Data represent the mean of percentage of BMP3 mRNA level ± standard deviation (SD) normalized to untreated cells (MG-63 T, HOS T).

Lian and Stein, 1992]. By Northern blot, using a riboprobe, we detected BMP3 mRNA in all human osteoblast cell lines and differentiated HBMSC at different level of expression 7 kb and 3 kb mRNA species were reported in fetal human tissues [Vukicevic et al., 1994]. In fetal rat calvaria osteoblasts, Chen et al. detected two different mRNA species (6 kb and 2 kb) [Chen et al., 1997]. However, in our study, Northern hybridization indicates only one major 6 kb mRNA species and a very weak and infrequently 3 kb mRNA species. By Northern blot, $1,25(OH)_2D_3$ treatment (10^{-8} M for 24 h) increases BMP3 mRNA expression in a few human osteoblast cell lines (MG-63, HOS) and HBMSC.

In differentiated HBMSC, BMP3 mRNA is weakly increased (115% ± 5). In these cells, $1,25(OH)_2D_3$ and BMP3 synergistically acted on osteocalcin (OC) synthesis, increasing highly the OC amount in culture medium [Amédée et al., 1994]. In many osteoblast cell cultures, $1,25(OH)_2D_3$ has been reported to stimulate the level of various bone-marker proteins [Spiess et al., 1986; Mahonen et al., 1990; Rao et al., 1996], and particularly the osteocalcin (OC), a bone-specific marker. In our experiments, OC is strikingly stimulated by $1,25(OH)_2D_3$ after 24 h of treatment and it seems an incubation for 48 h does not affect BMP3 expression.

BMP3 expression seems to be different in function of the development of tissues [Vukicevic et al., 1994]. In this paper, we used two states of differentiation: 1) HBMSC in differentiation process cultured without dexamethasone treatment, and 2) differentiated HBMSC with dexamethasone treatment, where OC content is high. In primary culture, without dexamathasone treatment, BMP3 stimulation is higher and is comparable to the stimulation obtained in HOS cells. In human cell lines, the level of BMP3 mRNA stimulation by

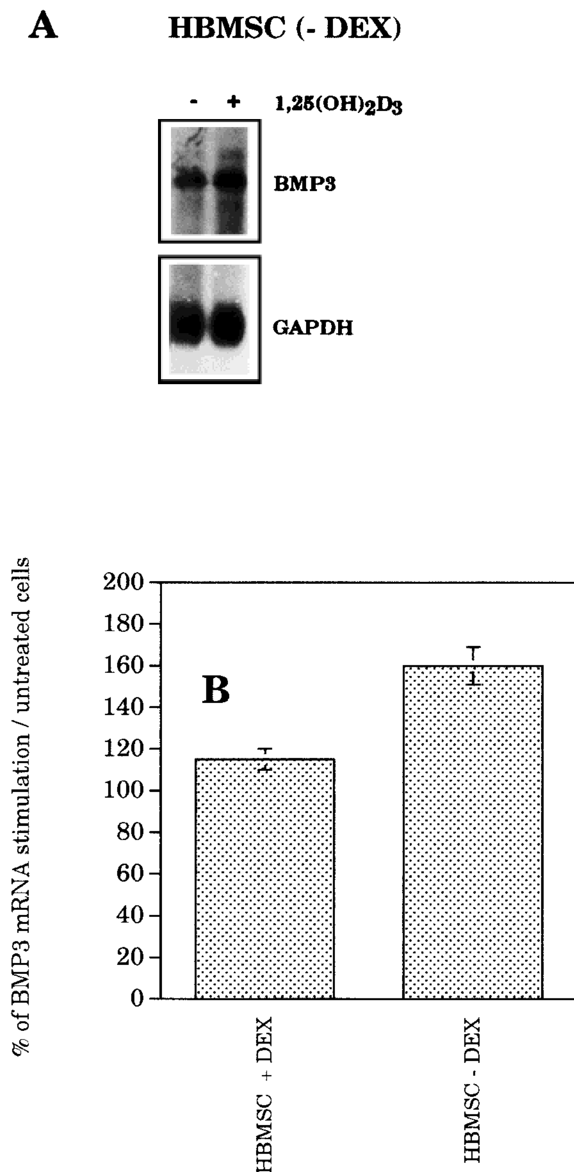


Fig. 4. Expression of BMP3 mRNA in primary HBMSC and 1,25$(OH)_2D_3$ regulation. Human bone marrow is plated at 2×10^7 cells/75 cm^2 and adherent cells are cultived for 2 weeks in IMDM + 10% FCS without dexamethasone treatment (−DEX) . Semi-confluent cells are treated (+) or not (−) with 1,25$(OH)_2D_3$ at 10^{-8} M for 24 h and extracted total RNA are used to perform Northern blot. **A**: Autoradiography of the blot. **B**: Signal intensity is quantified by OPTIMAS 5.2 programme as described in Materials and Methods. BMP3 expression is normalised to the GAPDH mRNA level (n = 2). Data represent the mean of percentage of stimulation ± standard deviation (SD) when compared to untreated cells. Percentage of BMP3 mRNA stimulation obtained in Figure 1 on differentiated cells treated with dexamethasone (+ DEX) was also reported in (B).

1,25$(OH)_2D_3$ is lower in comparison with OC mRNA stimulation. Particularly, a weak difference is detected between KHOS and MG-63 about BMP3 stimulation but OC level is strikingly stimulated in MG-63 compared to KHOS cells. In addition, BMP3 mRNA is stimulated two-fold in HOS cells but OC mRNA is not detected by Northern blot, whatever untreated or treated cells are in view of these results. It is difficult to assert that 1,25$(OH)_2D_3$ affect BMP3 expression in early states of differentiation because the exact level of differentiation is not identified in HBMSC. 1,25$(OH)_2D_3$ genomic regulation has been shown to be transcriptional and mediated by endogenous vitamin D_3 receptors (VDR) [Price and Baukol, 1980; Yoon et al., 1988; Kerner et al., 1989]. Then, 1,25$(OH)_2D_3$ effect on BMP3 expression could be related to the VDR level. As described previously [Faucheux et al., 1998], in absence of glucocorticoide treatment, these cells from the first to the third subculture expressed a low ALP activity, and OC was undetectable. However, both these undifferentiated and differentiated HBMSC expressed the vitamin D receptor (VDR) and are both sensitive to 1,25$(OH)_2D_3$ treatment. Then it is difficult to conclude that 1,25$(OH)_2D_3$ regulated mRNA BMP3 expression according to the vitamin D receptor level. Moreover, our results indicate that vitamin D3 response on mRNA BMP3 level was also related to the state on cell confluency. However, in this human cell culture model, RT-PCR analysis did not revealed no change in mRNA VDR expression, whatever the culture.

A synergism between BMPs and 1,25$(OH)_2D_3$ has been described by Yamaguchi et al. [1991]. In this paper, rhBMP2 strikingly induced osteocalcin mRNA and protein synthesis, only after 6 days of treatment in the presence of 1,25$(OH)_2D_3$ in C26 cell line, a rat potential osteoblast precursor cell line. 1,25$(OH)_2D_3$ may increase the bone-inductive activities of BMPs via mRNA stimulation.

A function of 1,25$(OH)_2D_3$ in osteoinduction could be assumed. VDR expression have been detected in embryonic skeleton at day 13 of rat fetus [Johnson et al., 1996]. In addition, 1,25$(OH)_2D_3$ increased MSX2 expression in human osteoblasts. This gene is homologous to Msx-2 (Hox8) which is particularly expressed in embryonic tissues to promote formation of skull bones and teeth in mouse [Hodgkinson et al., 1993]. Moreover, in our HBMSC model, we

detect higher increase of BMP3 mRNA expression after $1,25(OH)_2D_3$ treatment in proliferating cells and in the first subculture untreated with dexamethasone, than in confluent cells or differentiated cell cultures. An exact differentiation level is difficult to determine using primary cell cultures, but as far we know, in vitro culture provided a useful tool to obtain final differentiation step as a plateau which happens following the proliferation stage.

Our results seem to confirm an essential role of BMP3 in the differentiation process and in phenotype induction. This synergistic action between $1,25(OH)_2D_3$ and BMP3 may be important to stimulate bone formation and we do not exclude the possibility of interactions of other factors like TGFβ1 which are recently demonstrated in HBMSC model [Faucheux et al., 1997].

Growth factors have been shown to increase mRNA stability. The decrease of BMP3 mRNA level after cycloheximide (CHX) treatment in presence with $1,25(OH)_2D_3$ compared to cells treated with $1,25(OH)_2D_3$ alone shows that BMP3 stimulation is dependent on de novo protein synthesis.

$1,25(OH)_2D_3$ genomic action is mediated by VDR which recognized a vitamin D_3 response element (VDRE) [Kerner et al. 1989]. VDRE sequence was located to the promoter region of the human osteocalcin [Ozono et al., 1990] and others proteins as mouse calbindin-D28K [Takeda et al., 1994], rat calbindin-D9K [Darwish and De Luca, 1992], and mouse osteopontin [Noda et al., 1990]. To date, the mechanisms of $1,25(OH)_2D_3$ regulation on BMP3 expression are unknown. Actually, different hypothesis can be envisaged. $1,25(OH)_2D_3$ may act directly on BMP3 mRNA via promoter, or regulate transcriptional factors which may control BMP3 mRNA transcription. It is not excluded this synergism evenly implicates a reverse effect of BMP3 on $1,25(OH)_2D_3$ via receptors. BMP3 may affect transcriptional factors used for VDR expression or modify VDR affinity with the ligand. Thus, we set out to examine the following hypothesis concerning $1,25(OH)_2D_3$ effect on BMP3 expression: Does the BMP3 promoter contain the VDRE as observed for the osteocalcin gene?

ACKNOWLEDGMENTS

We are grateful to G.R. Mundy (San Antonio, TX) for providing the BMP3 cDNA. We thank S. Romberg and A.S. Virdi for helpful discussions and assistance. We also thank R.O.C. Oreffo for his assistance in the preparation of this manuscript. This work has been supported by MRC Bone Research Laboratory.

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