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Effects of Bone Morphogenetic Protein-2 on Human Neonatal Calvaria Cell Differentiation

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Abstract Bone morphogenetic proteins (BMPs) are factors that promote osteoblastic cell differentiation and osteogenesis. It is unknown whether BMPs may act on human osteoblastic cells by increasing immature cell growth and/or differentiation. We investigated the short- and long-term effects of recombinant human (rh)BMP-2 on cell growth and osteoblast phenotype in a new model of human neonatal pre-osteoblastic calvaria cells (HNC). In short-term culture, rhBMP-2 (20–100 ng/ml) inhibited DNA synthesis and increased alkaline phosphatase (ALP) activity without affecting osteocalcin (OC) production. When cultured for 3 weeks in the presence of ascorbic acid and inorganic phosphate to induce cell differentiation, HNC cells initially proliferated, type 1 collagen mRNA and protein levels rose, and then decreased, whereas OC mRNA and protein levels, and calcium accumulation into the extracellular matrix increased at 2 to 3 weeks. A transient treatment with rhBMP-2 (50 ng/ml) for 1 to 7 days which affected immature HNC cells, decreased cell growth, increased ALP activity and mRNA, and induced cells to express ALP, osteopontin, and OC at 7 days, as shown by immunocytochemistry. At 2 to 3 weeks, matrix mineralization was markedly increased despite cessation of treatment, and although OC and Col 1 mRNA and protein levels were not changed. A continuous treatment with rhBMP-2 for 3 weeks which affected immature and mature cells reduced cell growth, increased ALP activity and mRNA at 1 week and increased OC mRNA and protein levels and calcium content in the matrix at 3 weeks, indicating complete osteoblast differentiation. These results indicate that the differentiating effects of BMP-2 on human neonatal calvaria are dependent on duration of exposure. Although long-term exposure led to complete differentiation of OC-synthesizing osteoblasts, the primary effect of rhBMP-2 was to promote osteoblast marker expression in immature cells, which was sufficient to induce optimal matrix mineralization independently of cell growth and type 1 collagen expression. J. Cell. Biochem. 72:81–93, 1999. © 1999 Wiley-Liss, Inc.

Key words: human; calvaria; osteoblasts; BMP-2; differentiation

Bone morphogenetic proteins (BMPs) form a subgroup of factors in the transforming growth factor-β superfamily that play an important role in the initiation of bone formation [Wozney, 1992; Reddi, 1992; Urist, 1997]. BMPs stimulate bone formation by inducing the differentiation of mesenchymal cells into skeletal cells and by promoting the differentiation of cartilaginous/osteoblastic cells [Yamaguchi, 1995; Linkhart et al., 1996; Marie, 1997]. BMP-2, a prototype of the BMP subgroup [Wozney et al., 1988], induces a variety of effects on progenitor and skeletal cells. BMP-2 initiates the differentiation of multipotent mesenchymal progenitor cell lines to the osteogenic lineage, as assessed by induction of alkaline phosphatase (ALP) activity and expression of osteoblast markers, such as osteocalcin (OC) [Katagari et al., 1990; Yamaguchi et al., 1991; Thies et al., 1992; Ahrens et al., 1993]. In addition, BMP-2 promotes the maturation of committed cells to become more differentiated osteoblasts in several cell culture systems. For example, BMP-2 increases the expression of osteoblast marker genes and bone-like nodule formation in calvaria-derived cells [Chen et al., 1991; Hiraki et al., 1991; Takuwa et al., 1991; Ghosh-Choudhury et al., 1994; Hughes et al., 1995] and promotes the differentiation of bone marrow stromal cells into osteoblastic cells [Rickard et al., 1994; Lecanda et al., 1997; Fromigué et al., 1998]. Recent studies indicate, however, that the cellular response to BMPs may depend on cell maturation [Li et al., 1996]. In fetal rat calvaria cells, the regulation of bone matrix proteins by

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BMP-7 is dependent on the differentiated state [Li et al., 1996]. In pluripotent mesenchymal cell cultures, osteoblast marker expression and matrix mineralization are also dependent on the duration of exposure to BMP-2 [Puleo, 1997]. It is unknown, however, whether BMPs may promote human osteoblastic cell differentiation by increasing the growth and/or differentiation of cells at different stages of maturation.

In this study, we investigated the effects of BMP-2 and the cellular response of human osteoblastic cells using a new model of human neonatal calvaria cell cultures with characteristics of pre-osteoblasts [de Pollak et al., 1996, 1997; Lomri et al., 1997a; Debiais et al., 1998]. We report here that the promoting effects of BMP-2 on human calvaria cell phenotype and osteogenesis are dependent on the duration of exposure and that the optimal osteogenic effect of rhBMP-2 is related to its action on immature HNC cells, independently of changes in cell growth.

MATERIALS AND METHODS

Cell Cultures

Human calvaria bone samples consisting of normal bone samples surrounding sutures were obtained from nine neonates, aged 4–7 months, during local surgery, according to the French ethical recommendations [de Pollak et al., 1996, 1997]. The samples were sectioned under sterile conditions and human calvaria cells were isolated by collagenase digestion, as described previously [de Pollak et al., 1997]. Briefly, after washing in PBS, the samples were dissected into 1 mm^3 size fragments and treated with 0.25% collagenase (Type I, Sigma, St. Louis, MO) for 2 h at 37°C. The collagenase-treated bone fragments were then washed extensively with Dulbecco's modified essential medium (DMEM) supplemented with glutamine (292 mg/L), 10% heat inactivated fetal calf serum (FCS), and 1% antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin). The cells were collected by centrifugation, suspended in DMEM, plated in 25 cm^2 flasks and cultured in DMEM supplemented with 10% FCS at 37°C. At confluence, the cells were detached with 0.1 % trypsin/EDTA, counted, and plated at the indicated density. All cell populations (only first to third passaged cells) were cultured under identical conditions. The cell population obtained using this method was composed mostly of undifferentiated cells showing characteristics of pre-osteoblasts [de Pollak et al., 1997, Lomri et al., 1997a, Debiais et al., 1998].

Cell Proliferation

To determine the effects of BMP-2 on cell growth, human neonatal calvaria (HNC) cells were plated at 10,000 cells/cm^2 in 24-well plates, in DMEM plus 10% FCS. At subconfluence, the cells were cultured for 24 h in DMEM with 1% FCS, then were treated with recombinant human (rh)BMP-2 (kindly provided by Genetics Institute, Cambridge, MA), or the solvent, at the indicated concentration. A time course study was also performed from 1 to 6 days of culture. The cells were labeled with 2 µCi/well of 6-[^{3}H]-thymidine for 4 h before the end of treatment, collected by trypsinization, and DNA was precipitated with thrichloracetic acid (TCA). The TCA-insoluble fraction was dissolved in NaOH and [^{3}H]-thymidine incorporation into DNA was measured by liquid scintillation [de Pollak et al., 1997].

Osteoblast Markers

To evaluate the effects of BMP-2 on osteoblast markers, HNC cells were plated at 10,000 cells/cm^2 in six-well plates in DMEM with 10 % FCS. At confluence, the cells were incubated in DMEM plus 1% FCS for 24 h. Then the cells were cultured in DMEM plus 1% FCS, 0.1 % BSA, 50 µg/ml ascorbic acid, and 50 µg/ml vitamin K [de Pollak et al., 1997] and were treated with rhBMP-2, or the solvent, at the indicated concentration. A time-course study was also performed from 1 to 4 days. At each time-point, the cells were rinsed with cold PBS, scraped in distilled water, sonicated, and ALP activity in the cell lysates was determined by a colorimetric method using phenyl phosphate as substrate (phosphatase alcaline kit bioMerieux, France). The protein content of the cell lysates was determined colorimetrically (Bio-Rad Protein Assay, France), and the activity of the enzyme was expressed as nanomoles of p-nitrophenol released per min/mg protein. At the end of treatment, the medium was removed and frozen. Osteocalcin concentration in the medium was measured in duplicate by RIA (Cis-Oris, Gif sur Yvette, France) using a specific antibody raised against bovine osteocalcin. The maximum inter- and intra-assay coefficients of variation for the range of concentrations evaluated are 6.6% and 3.7%, respec-

tively, and the lower limit of detection of the assay is 0.35 μg/L [de Pollak et al., 1997]. The data were corrected for osteocalcin levels in the serum.

The effects of rhBMP-2 on the cell production of type 1 collagen (Col 1) was evaluated by determining the levels of carboxyterminal propeptide (P1CP) released into the medium. P1CP levels were measured with a procollagen [^{125}I]-RIA kit using a specific antibody (Orion Diagnostic., Espoo, Finland) and the results were expressed as μg P1CP/mg protein. The intra- and inter-assay variability of the assay are 3.2% and 4.0%, respectively.

Immunocytochemistry

To determine whether rhBMP-2 stimulated human calvaria cell differentiation by increasing the number of immature cells, human calvaria cells were treated with 50 ng/ml rhBMP-2 for 4 to 7 days and the expression of ALP and bone matrix proteins was determined by cytochemistry or immunocytochemistry using specific rabbit polyclonal antibodies against osteopontin (LF-6) and osteocalcin (LF-32; kindly provided by Dr. L. Fisher, NIH, Bethesda, MD) used at 1/100 dilution, as described previously [de Pollak et al., 1996, 1997].

Cell Differentiation in Long-Term Culture

To determine the effects of BMP-2 on human calvaria cell differentiation in long-term culture, HNC cells were plated at high density (25,000 cells/cm^2) in 24-well plates and cultured for 21 days in the presence of 10% FCS, 50 μg/ml ascorbic acid, 50 μg/ml vitamin K, and 3 mM inorganic phosphate, conditions that induce the differentiation of rat [Bellows et al., 1986; Stein and Lian, 1993] and human calvaria cells [de Pollak et al., 1997; Debiais et al., 1998]. In these conditions, the cells first proliferated, then become more mature and formed a mineralized matrix that became mineralized progressively with time in culture. To determine the effects of short- and long-term exposure to rhBMP-2, HNC cells were treated with rhBMP-2 at an optimal effective dose (50 ng/ml) at two different periods of culture. In the first series of experiments, the cells were treated continuously during 3 weeks. In the second series, the cells were treated with rhBMP-2 only during the first period of culture (day 1–7 of culture) when cells are still immature. In each experiment, parameters of cell growth and differentiation were determined at the indicated time-points. DNA content used as a reflection of cell growth was evaluated by fluorometric analysis [West et al., 1985], and markers of cell differentiation (ALP activity, OC production and P1CP levels) were determined at 1, 2, and 3 weeks, as described above.

To confirm the biochemical evaluation of ALP, an histochemical staining of the enzyme was performed. HNC cells were rinsed with PBS, fixed in 70% ethanol at 4°C, and incubated for 1 h at 37°C with naphtol AS-BI phosphate in Tris-buffer (pH 8.5) in the presence of Fast red violet LB salt). Calcium incorporation into the matrix was visualized by microscopic analysis. The incorporation of calcium into the matrix was also evaluated biochemically. The cells were washed with cold PBS, scraped, centrifuged, and the pellets were dissolved in 10% formic acid for 24 h at room temperature. Total calcium content in the matrix was determined by a colorimetric assay using a Ca-kit (bioMerieux, France) [Fromigué et al., 1997].

RT-PCR Analysis

To evaluate the effects of rhBMP-2 on differentiation marker genes, the expression of ALP, Col 1, and OC mRNAs after continuous and transient treatments with rhBMP-2 was analyzed by reverse transcription-polymerase chain reaction (RT-PCR), as described previously [Lomri et al., 1997a; Fromigué et al., 1997]. Optimization of RT-PCR results was carried out by generating saturations curves of RT-PCR products of ALP, α1(I)Col 1, OC and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) against cycle number (0–35 cycles) in order to allow semi-quantitative variation of product levels. We choose the cycle number (30 cycles) in which the amplification was linear for all genes. After 4, 7, 14, or 19 days of culture in the presence of rhBMP-2, HNC cells were washed with PBS and Lysol with the Extract-All (Eurobio, France) reagent according to the manufacturer's instructions. Three μg total cellular RNA from each samples were reverse transcribed and the cDNA samples were then divided and amplified using specific primers for ALP, α1(I)Col 1, OC, and GAPDH whose sequences and PCR conditions were previously reported in detail [Fromigué et al., 1997]. Southern blots were performed by running aliquots of amplified cDNA on 1.2% agarose gel followed by transfer onto nylon membrane according to the

manufacturer's protocol. Hybridization of blots was carried out overnight at 50°C with [γ^{32}P]ATP labeled internal primers. Membranes were washed twice in 2 × SSC/0.1% SDS at room temperature for 10 min, once in 0.1 × SSC/0.1% SDS at 50°C for 10 min, then the filters were exposed to X-ray films. Autoradiographic signals were quantified using a scanning densitometer (Transydine General Corporation, Ann Arbor, MI). The signal for each gene was corrected for GAPDH.

Statistical Analysis

The data are expressed as the mean ± SEM and were analyzed using the statistical package super-ANOVA (Macintosh, Abacus concepts, Inc., Berkeley, CA). Differences between the mean values were evaluated with a minimal significance of $P < 0.05$.

RESULTS

Effects of rhBMP-2 on HC Cell Growth and Osteoblast Markers

Since no information was available on BMP-2 effects on human calvaria cells, we first determined the optimal effective dose and time course effects of rhBMP-2 on HNC cell proliferation and markers of differentiation. Treatment of HNC cells with rhBMP-2 (0.1–100 ng/ml, 3 days) dose dependently decreased cell growth, as evaluated by (^{3}H)-thymidine incorporation into DNA, with a maximal effect at 100 ng/ml (Fig. 1A). The time-course study showed that the inhibitory effect of rhBMP-2 (50 ng/ml) on cell growth was observed at 3 to 6 days of culture (Fig. 1B). Treatment of human calvaria cells with rhBMP-2 dose-dependently increased ALP activity with a maximal effect at 50 ng/ml (Fig. 2A). This effect occurred at 2 to 4 days of treatment (Fig. 2B). In contrast, rhBMP-2 (0.1–100 ng/ml, 48–96 h) had no effect on osteocalcin production or on collagen type 1 synthesis, as evaluated by P1CP levels in the medium in short term culture (Table 1). These results show that rhBMP-2 decreased HNC cell growth and increased ALP activity but did not affect Col 1 synthesis and OC production in short-term culture.

Effects of Continuous Treatment With rhBMP-2

Human calvaria cells were induced to differentiate in long-term culture in the presence of ascorbic acid and phosphate. In this model, human calvaria cells first proliferated, expressed ALP activity and synthesized type 1 collagen, thereafter ALP activity and collagen synthesis decreased whereas the extracellular matrix accumulated and become mineralized. Osteocalcin expression and calcium incorporation into the extracellular matrix increased with time in culture, as depicted in Figure 3. This pattern presents some similarities with the temporal variations previously observed in rat calvaria cells [Stein and Lian, 1993]. We then investigated the effects of short- and long-term exposures to rhBMP-2 on HC cells. In the first

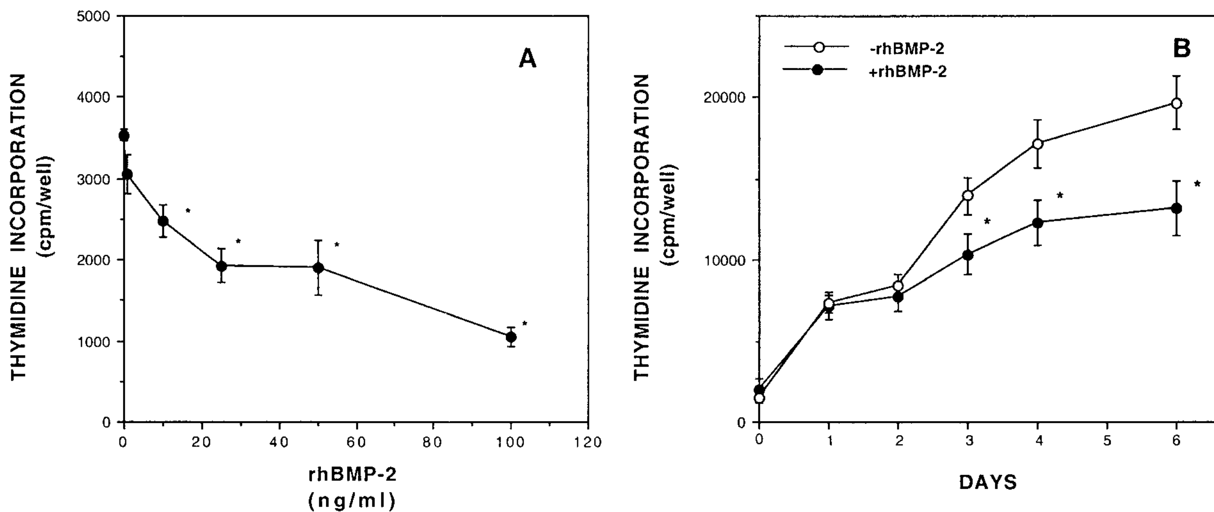

Fig. 1. Inhibitory effect of rhBMP-2 on DNA synthesis in human calvaria cells in short-term cultures. **A**: The dose-dependent effect of rhBMP-2 at 3 days. **B**: The time-course effect of rhBMP-2 (100 ng/ml). Data are the mean ± SEM of three to four values (*$P < 0.01$ vs. untreated cells).

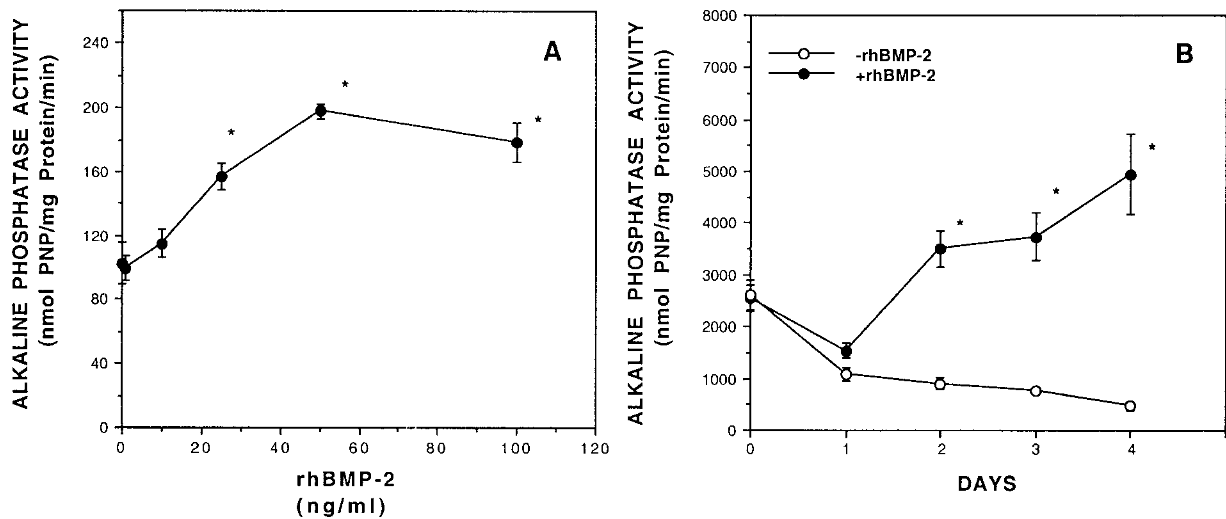

Fig. 2. Stimulatory effect of rhBMP-2 on alkaline phosphatase activity in human calvaria cells in short-term culture. **A**: The dose-dependent effect of rhBMP-2 (48 h). **B**: The time-course effect of rhBMP-2 (100 ng/ml). Data are the mean ± SEM of three to four values (*$P < 0.01$ vs. untreated cells).

TABLE I. Lack of Effect of rhBMP-2 on Osteocalcin Production and Type 1 Collagen Synthesis as Evaluated by P1CP Levels in the Medium in Human Neonatal Calvaria Cells in Short-Term Culture[a]

Treatment	P1CP levels (ng/mg protein)	Osteocalcin (ng/mg protein)
Control	6.10 ± 0.49	6.59 ± 1.28
rhBMP-2	7.38 ± 1.33	8.88 ± 1.01

[a]Confluent cells were treated with 50 ng/ml rhBMP-2 for 2 days. The data are the mean ± SEM of three to four values.

series of experiments, the cells were treated continuously in order to test the effects on immature cells (Fig. 3). The continuous treatment for 21 days with rhBMP-2 (50 ng/ml) slightly decreased DNA content by 19%, 25%, and 45% (all $P < 0.05$ compared to controls) at 1, 2, and 3 weeks of culture, respectively (not shown). This confirms the inhibitory effect on cell growth observed in short-term culture. In contrast, the continuous treatment increased ALP activity with a peak at 1 week of culture (Fig. 4A). In addition, the continuous treatment with rhBMP-2 increased OC production by HNC cells at late time points in culture (2–3 weeks; Fig. 5A). However, P1CP levels in the medium were not affected (Fig. 5B).

The continuous treatment of HNC cells with rhBMP-2 increased calcium levels in the mineralized matrix formed by HC cells. This effect was apparent at 2 weeks of culture when calcium deposition in the matrix was visible morphologically (not shown). The stimulatory effect of continuous rhBMP-2 on matrix mineralization was also observed biochemically at 2 weeks of culture (Fig. 6). These results indicate that the continuous treatment with rhBMP-2 increased transiently ALP activity, promoted the differentiation of HNC cells into more mature osteoblastic cells producing osteocalcin at 2 to 3 weeks of culture, and increased matrix mineralization.

Effects of Transient Treatment With rhBMP-2

To determine whether rhBMP-2 affected differently immature and mature HNC cells, the cells were treated with rhBMP-2 transiently during the first week of culture when immature cells are proliferating, as depicted in Figure 3.

As found with the continuous treatment, the transient treatment with rhBMP-2 during day 1–7 of culture decreased DNA content by 18% ($P < 0.05$) at 1 week (not shown). This effect was transient as it was not found after treatment withdrawal. On the other hand, the transient treatment with rhBMP-2 induced a marked and transient increase in ALP activity at 1 week, which was similar to the effect of the continuous treatment, except that ALP activity returned to control levels after treatment withdrawal (Fig. 4B). The cytochemical analysis showed that the increased ALP activity induced by rhBMP-2 was related to an increased number of ALP-positive cells at 4 and 7 days of treatment (Fig. 7, Table 2). Furthermore, the

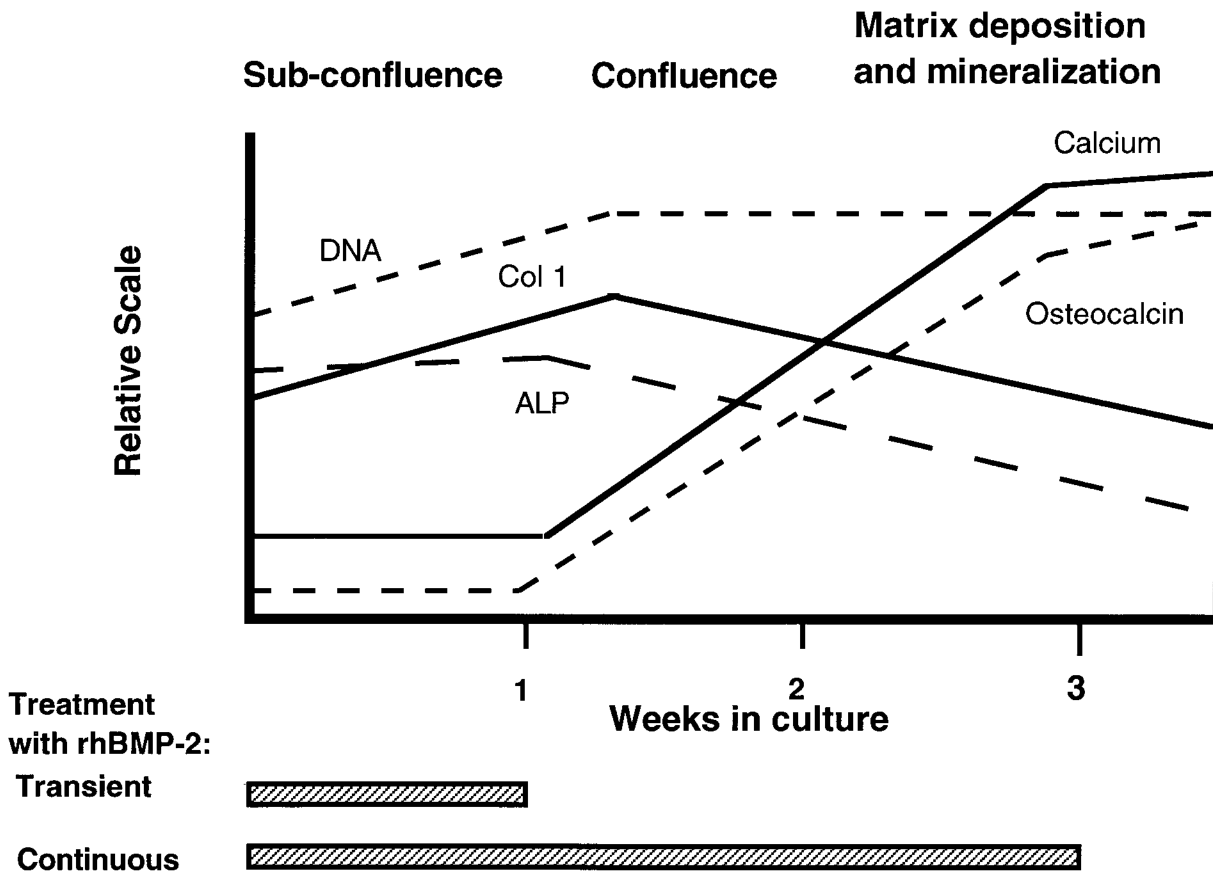

Fig. 3. Temporal sequence of changes of cell growth and osteoblast markers during human calvaria cell differentiation in vitro and experimental protocols of treatments with rhBMP-2. The pattern represents the relative changes in alkaline phosphatase (ALP) mRNA and activity, and in collagen type 1 (Col 1) and osteocalcin (OC) mRNAs and proteins. When induced to differentiate in the presence of 10% serum, ascorbic acid, and inorganic phosphate, HNC cells first proliferate, as evaluated by DNA content, Col 1 initially increases then decreases, thereafter ALP decreases and OC expression and calcium content in the matrix increase during the late stages of differentiation. HNC cells were treated transiently or continuously with rhBMP-2 to affect immature HNC cells or all cells, respectively.

immunocytochemical analysis showed that rhBMP-2 induced more cells expressing osteopontin and osteocalcin at 7 days of culture (Table 2), indicating that rhBMP-2 rapidly induced the expression of markers of the osteoblast phenotype in immature HNC cells.

The transient treatment with rhBMP-2 markedly increased calcium levels in the mineralized matrix formed by HNC cells. This was evidenced by the increased black deposits visualized by von Kossa staining in the matrix (Fig. 7) and was also confirmed biochemically (Fig. 6). The transient treatment with rhBMP-2 increased matrix mineralization more than the continuous treatment and for up to 3 weeks of culture despite cessation of treatment, showing that the transient treatment was more efficient to promote matrix mineralization than the continuous treatment (Fig. 6). In contrast, the transient treatment with rhBMP-2 did not induce the production of osteocalcin at 2 to 3 weeks (Fig. 5A), suggesting that this short treatment did not induce the complete differentiation of immature cells into OC-synthesizing cells. As for the continuous treatment, the increased matrix mineralization induced by the transient rhBMP-2 treatment was unrelated to the effect on type 1 collagen synthesis evaluated by P1CP levels (Fig. 5B). These results show that a transient stimulation with rhBMP-2 induces immature HNC cells to differentiate and induces a marked stimulatory effect on matrix mineralization despite cessation of treatment.

Time-Dependent Effect of rhBMP-2 on Osteoblast Marker Gene Expression

To further investigate the effects of transient and continuous treatments with rhBMP-2 on

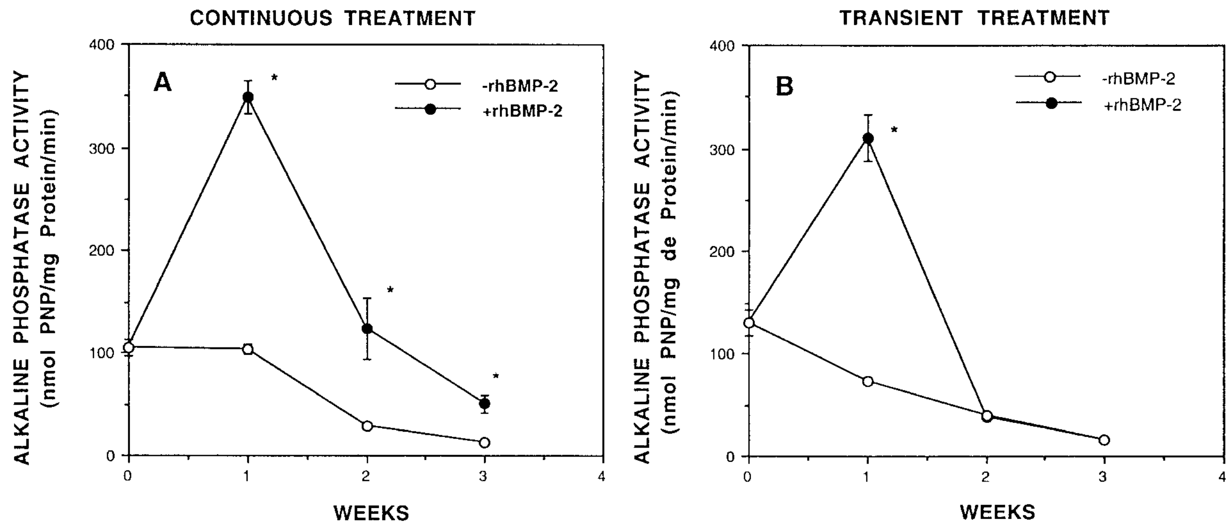

Fig. 4. Stimulatory effect of continuous (1–21 days) (**A**) and transient treatments (1–7 days) (**B**) with rhBMP-2 (50 ng/ml) on ALP activity during HNC differentiation in long term culture. Data are the mean of three to four values ± SEM (*$P < 0.05$ vs. untreated cells).

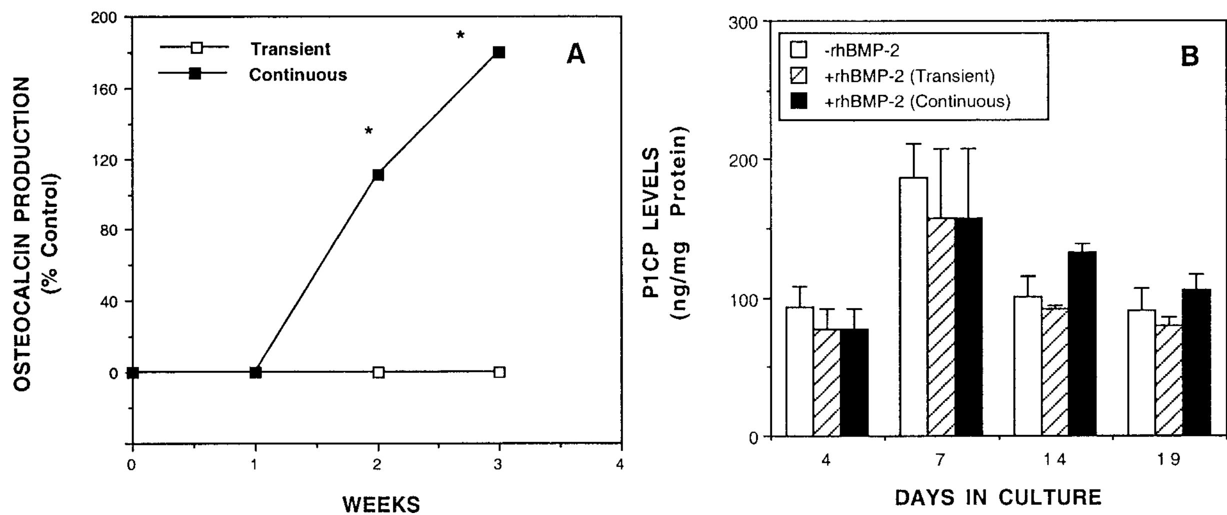

Fig. 5. Effects of continuous (1–21 days) and transient treatments (1–7 days) with rhBMP-2 (50 ng/ml) on type 1 collagen synthesis evaluated by P1CP (**A**), and osteocalcin (**B**) levels released during HNC differentiation in long term-culture. Data are the mean of three to four values ± SEM (*$P < 0.05$ vs. untreated cells).

human calvaria, the expression of ALP, Col 1, and OC mRNAs was investigated. Figure 8 shows that the continuous and transient treatments with rhBMP-2 induced a marked increase in ALP mRNA levels, as evaluated by semi-quantitative RT-PCR. The amplitude and time-course of ALP mRNA were similar in the two treatments and comparable to the elevation in ALP activity (Fig. 4). As also shown in Figure 8, the continuous and transient treatments with rhBMP-2 had no effect on Col 1 mRNA levels, confirming the results obtained with P1CP levels (Table 1). The analysis of OC mRNA showed that the continuous treatment of HC cells with rhBMP-2 increased OC mRNA expression at 2 to 3 weeks whereas the transient treatment had no significant effect (Fig. 8), confirming the biochemical analysis of OC synthesis. Altogether, these data indicate that rhBMP-2 acted differentially on osteoblast marker genes in immature and more mature human calvaria cells. Short-term exposure to rhBMP-2 induced immature HC cells to differentiate into osteoblastic cells, whereas long term exposure led to the complete differentiation into mature OC-synthesizing osteoblasts. However,

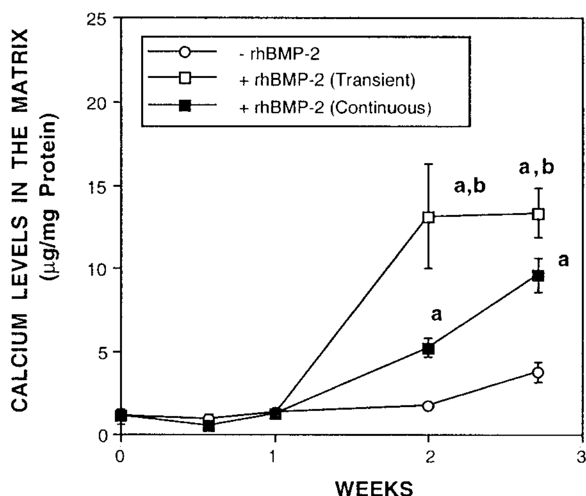

Fig. 6. Distinct effects of continuous (1–3 weeks) and transient treatment (1–7 days) with rhBMP-2 (50 ng/ml) on calcium content in the extracellular matrix formed by HNC cells during differentiation in long-term culture. Data are the mean ± SEM of three to four values (a,b: $P < 0.05$ vs. untreated cells and continuous treatment, respectively).

short-term exposure to rhBMP-2 was sufficient to promote matrix mineralization in this model, independently of cell growth and type 1 collagen expression.

DISCUSSION

The recent development of human calvaria cell cultures [de Pollak et al., 1996, 1997] allowed us to investigate the differential mechanisms of action of local factors regulating human calvaria osteogenesis [Lomri et al., 1997a; Debiais et al., 1998]. In the present study, we show that the differentiating effects of rhBMP-2 on human calvaria are dependent on duration of exposure and that rhBMP-2 promoted osteoblast differentiation by acting on immature cells, which resulted in optimal matrix mineralization.

Since BMPs may promote osteogenesis by stimulating the growth and/or differentiation of osteoblast precursor cells [Katagiri et al., 1990; Hiraki et al., 1991; Knutsen et al., 1990; Sampath et al., 1992; Vukicevik et al., 1990; Yamaguchi et al., 1996], including in humans [Amédée et al., 1994; Fromigué et al., 1998], we initially determined whether rhBMP-2 affected cell growth in the human calvaria cell system. Our data indicate that rhBMP-2 inhibited human calvaria cell proliferation in both short- and long-term cultures, which is consistent with the inhibitory effect of BMP-2 on cell growth in rat calvaria cells [Iwasaki et al., 1994]. BMP-2 and BMP-3 were also found to inhibit cell proliferation in mouse calvaria-derived MC3T3-E1 cells [Vukicevik et al., 1990]. In contrast, BMP-3 does not appear to affect rat calvaria cell growth [Vukicevik et al., 1989] whereas BMP-7/0P-1 [Knutsen et al., 1990; Sampath et al., 1992; Li et al., 1996] and BMP-4 [Chen et al., 1991] increase rat calvaria cell growth. The promoting effect of BMP-2 on osteogenesis in human calvaria cell cultures was not related to an increased cell growth, but was associated with increased ALP activity in both short- and long-term cultures, which is consistent with previous data in calvaria or marrow stroma-derived cells [Iwasaki et al., 1994; Rickard et al., 1994; Takuwa et al., 1991; Yamaguchi et al., 1996; Fromigué et al., 1998]. This indicates that the primary effect of rhBMP-2 on human calvaria cells is to promote the differentiation of immature cells into osteoblasts rather than to increase the growth of osteoblast precursor cells in HNC cultures.

We then determined whether the differentiating effects of rhBMP-2 on human calvaria cells are related to duration of exposure. HNC cells were cultured in the presence of ascorbic acid and phosphate, conditions that were shown to induce osteoblast differentiation and osteogenesis in rat [Bellows et al., 1991; Stein et al., 1993] and human [de Pollak et al., 1997; Debiais et al., 1998] calvaria cells. In this model, we identified a temporal sequence of events characterized by initial cell proliferation followed by matrix synthesis, deposition, and mineralization. This pattern presents similarities with the sequence previously characterized in the rat calvaria system [Stein and Lian, 1993]. During the first phase, HNC cells first proliferated and type 1 collagen production increased, then ALP activity and P1CP levels fell and calcium levels in the mineralized matrix rose, showing that HNC cell proliferation was followed by progressive cell differentiation and matrix mineralization (Fig. 3). This sequence of events reflects the temporal changes in proliferation and the subsequent differentiation of human neonatal calvaria cells observed in vivo [de Pollak et al., 1996]. This model is therefore useful to analyze the proliferation and differentiation of human calvaria osteoblasts under stimulation with local anabolic agents [Debiais et al., 1998]. In this system, we compared the effects of a continuous treatment with rhBMP-2,

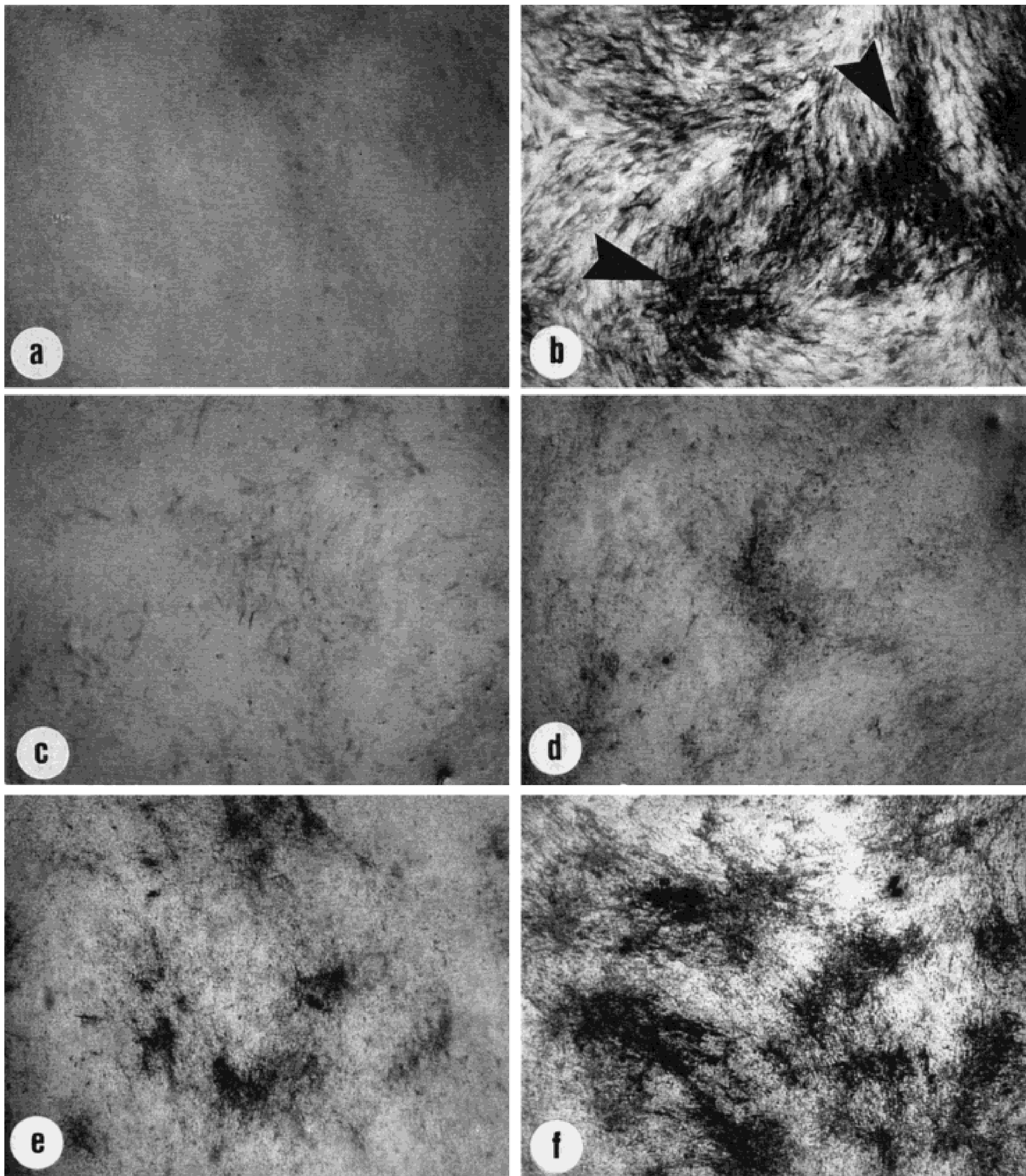

Fig. 7. Microphotographs showing the stimulatory effect of a transient treatment (1–7 days) with rhBMP-2 (50 ng/ml) on alkaline phosphatase activity (**a**,**b**) and calcium (**c–f**) incorporated into the matrix formed by HNC cells cultured in the presence of ascorbic acid and phosphate for 1–3 weeks. The transient treatment increased alkaline phosphatase (arrows) activity at 1 week (b vs. a) and calcium uptake (appearing in black) in the matrix at 2 (d vs. c) and 3 weeks (f vs. e). Original magnification: ×25.

which affected both immature and mature cells, and a transient treatment which affected only immature cells. The continuous and transient treatments with rhBMP-2 induced a rapid increase in ALP activity and mRNA levels. However, this effect was transient, even during a continuous exposure with rhBMP-2, suggesting that rhBMP-2 rapidly induced the differentiation of pre-osteoblastic cells. This was confirmed by our finding that rhBMP-2 increased

TABLE II. Early Induction of Osteoblast Characteristics by rhBMP-2 in Human Neonatal Calvaria Cells[a]

	Day 0	Day 4		Day 7	
Marker	Control	Control	Treated	Control	Treated
ALP+	3.3 ± 0.3	7.6 ± 0.7	14.8 ± 0.8*	16.4 ± 0.4	24.9 ± 0.4*
OP+	0	0	0	0	5.1 ± 0.3*
OC+	0	0	0	0	5.3 ± 0.5*

[a]Cells were treated with 50 ng/ml rhBMP-2 in the presence of ascorbic acid. The data are the mean ± SEM of three to four values, and are expressed as % of total cells showing a positive immunolabeling for the marker.
*Indicates a significant difference with control cells ($P < 0.05$).

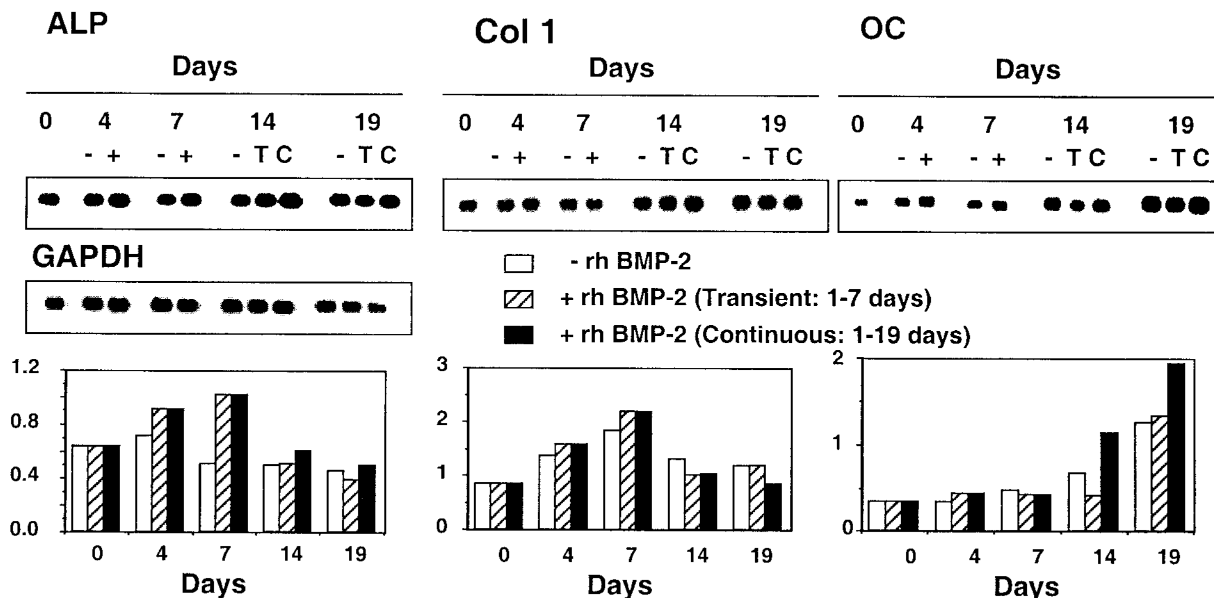

Fig. 8. Distinct effects of continuous and transient treatments with rhBMP-2 (50 ng/ml) on alkaline phosphatase (ALP), type 1 collagen (Col 1), and osteocalcin (OC) mRNA levels in HNC cells during differentiation in long-term culture. The cells were treated for 7 days (Transient treatment) or 19 days (Continuous treatment) and gene expression was evaluated by RT-PCR analysis. The figure shows autoradiograms of amplified cDNA hybridized to [γ^{32}P]-labelled antisense internal oligoprobes, and the signal for each gene was normalized to GAPDH.

the number of ALP-positive cells, as well as the number of osteocalcin- and osteopontin-positive cells, showing that rhBMP-2 rapidly induced the commitment of undifferentiated HC cells to become cells expressing the osteoblast phenotype.

The expression and secretion of osteoblast markers was influenced differentially by the duration of exposure to rhBMP-2. While transient (7 days) and continuous (21 days) exposure to rhBMP-2 increased transiently ALP activity and mRNA, the transient treatment with rhBMP-2 did not affect osteocalcin expression after 7 days. In contrast, the continuous treatment induced a permanent increase in OC production and mRNA levels at late time points. Thus, the continuous presence of rhBMP-2 for more than 1 week was required for the induction of OC by HNC cells. In rat [Puleo, 1997] and human [Fromigué et al., 1998] bone marrow stromal cells, the continuous exposure of cells to BMP-2 is also required for OC expression. Similarly, BMP-2 and other BMPs such as BMP-4 and BMP-6 increased OC expression at late time-points in rat calvaria cells [Ghosh-Choudhury et al., 1996; Li et al., 1996]. Since rhBMP-2 increased the number of OC-positive NHC cells with time, the rise in OC mRNA and protein levels induced by rhBMP-2 in long-term HNC culture resulted from the progressive differentiation of pre-osteoblastic cells into mature cells expressing osteocalcin. Thus, in the present model composed mostly of undifferentiated human calvaria cells, the primary effect of

rhBMP-2 was to induce the differentiation of immature cells into pre-osteoblasts which then differentiate into osteoblasts under long-term exposure to BMP-2.

With regards to bone matrix proteins, we found that rhBMP-2 had no effect on type 1 collagen mRNA levels and production by HNC cells in short- and long-term cultures. In contrast, BMP-2, -3, -4, or-7 were found to increase collagen synthesis in rat calvaria cells [Vukicevik et al., 1989; Chen et al., 1991; Knutsen et al., 1991; Sampath et al., 1992; Li et al., 1996]. Although our data indicate that BMP-2 did not alter type 1 collagen synthesis by NHC cells, we did not evaluate the amount of collagen deposited in the matrix. The finding that rhBMP-2 did not alter the expression of type 1 collagen in long-term culture suggests that the stimulatory effect of BMP-2 on matrix mineralization was independent of changes in type 1 collagen expression. In rat calvaria cells, matrix mineralization induced by BMP-2 is also independent of bone matrix protein synthesis [Li et al., 1996]. In addition, we found that the transient treatment with rhBMP-2 was more efficient than the continuous treatment to stimulate matrix mineralization. This differs from the rat bone marrow stromal system in which the longer the cells were exposed to BMP-2, the greater was the mineral incorporation into the matrix [Puleo, 1997]. In this system, however, the stimulatory effect of BMP-2 reflects an increased number and size of nodules composed of mineralized matrix. In contrast, collagen synthesis was unaffected by rhBMP-2 in HNC cultures. Thus, the lower matrix mineralization induced by the continuous treatment with rhBMP-2 does not reflect a decreased matrix synthesis, and may result from the negative effect of rhBMP-2 on cell growth in long-term culture.

The finding that rhBMP-2 affected differently osteoblast marker genes and matrix mineralization in immature and more mature HNC cells may suggest that these effects are differentiation stage specific. In multipotential calvaria-derived cell lines, BMP-2 was previously found to exert cell differentiation-dependent effects [Yamaguchi et al., 1991]. In rat calvaria cells, the effects of BMP-7/OP-1 was also found to be differentiation stage specific [Li et al., 1996]. Several mechanisms may account for the distinct response of immature and more differentiated human calvaria cells to rhBMP-2. HNC cells may express distinct types of BMP receptors at different stages of differentiation, as reported in rat calvaria cells [Centrella et al., 1995]. It is also possible that BMP-2 acted distinctly at different steps during the sequence of human calvaria cell differentiation by inducing the production of several factors such as Insulin Like Growth Factors [Canalis and Gabbitas, 1993; Yeh et al., 1996] or BMPs [Ghosh-Coudhury et al., 1994; Honda et al., 1997]. Thus, multiple mechanisms may be involved in the differential effects of rhBMP-2 on human calvaria cells at different stages of maturation.

In addition to provide the first insight into the stage-dependent effects of rhBMP-2 on human calvaria cell differentiation, the present study indicates that immature cells in this system are the main target cells of rhBMP-2. As our results indicate that the optimal osteogenic effect of rhBMP-2 is related to its action on immature HNC cells, it will be of interest to delineate the early mechanisms of action of rhBMP-2 in these cells. The recent development of human calvaria cell lines in our laboratory [Lomri et al., 1997b] will provide a basis to determine the cellular and molecular mechanisms that are involved in the promotion of human calvaria osteogenesis by rhBMP-2.

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