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ARTICLES

Bone Morphogenetic Protein-2 and Transforming Growth Factor-β_2 Interact to Modulate Human Bone Marrow Stromal Cell Proliferation and Differentiation

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Abstract Osteoprogenitor cells in the human bone marrow stroma can be induced to differentiate into osteoblasts under stimulation with hormonal and local factors. We previously showed that human bone marrow stromal (HBMS) cells respond to dexamethasone and vitamin D by expressing several osteoblastic markers. In this study, we investigated the effects and interactions of local factors (BMP-2 and TGF-β_2) on HBMS cell proliferation and differentiation in short-term and long-term cultures. We found that rhTGF-β_2 increased DNA content and stimulated type I collagen synthesis, but inhibited ALP activity and mRNA levels, osteocalcin production, and mineralization of the matrix formed by HBMS cells. In contrast, rhBMP-2 increased ALP activity and mRNA levels, osteocalcin levels and calcium deposition in the extracellular matrix without affecting type I collagen synthesis and mRNA levels, showing that rhBMP-2 and rhTGF-β_2 regulate differentially HBMS cells. Co-treatment with rhBMP-2 and rhTGF-β_2 led to intermediate effects on HBMS cell proliferation and differentiation markers. rhTGF-β_2 attenuated the stimulatory effect of rhBMP-2 on osteocalcin levels, and ALP activity and mRNA levels, whereas rhBMP-2 reduced the rhTGF-β_2-enhanced DNA synthesis and type I collagen synthesis. We also investigated the effects of sequential treatments with rhBMP-2 and rhTGF-β_2 on HBMS cell differentiation in long-term culture. A transient (9 days) treatment with rhBMP-2 abolished the rhTGF-β_2 response of HBMS cells on ALP activity. In contrast, a transient (10 days) treatment with rhTGF-β_2 did not influence the subsequent rhBMP-2 action on HBMS cell differentiation. The data show that TGF-β_2 acts by increasing HBMS cell proliferation and type I collagen synthesis whereas BMP-2 acts by promoting HBMS cell differentiation. These observations suggest that TGF-β_2 and BMP-2 may act in a sequential manner at different stages to promote human bone marrow stromal cell differentiation towards the osteoblast phenotype. J. Cell. Biochem. 68:411–426, 1998.

Key words: bone marrow stroma; human; differentiation; TGF-β; BMP-2

Bone marrow stroma contains a population of multipotent stem cells that can differentiate into several separate lineages, including fibroblasts, adipocytes, reticular cells, and osteogenic cells [Friedenstein, 1976; Owen, 1988]. We and others have reported that human bone marrow stromal (HBMS) cells can be induced to differentiate into cells expressing alkaline phosphatase (ALP) activity, osteocalcin (OC) and type I collagen (Col I), and give rise to mineralizing cultures under stimulation with growth factors or hormones [Beresford et al., 1994; Cheng et al., 1994; Long et al., 1995; Fromigué et al., 1997]. However, in contrast to animal cells, human bone marrow stromal cell cultures do not produce bone-like nodular structures in vitro [Cheng et al., 1994; Fromigué et al., 1997]. The complete induction of osteogenesis may require the effect of multiple local factors acting in a coordinated fashion to control cell proliferation and generate cell differentiation through a multi-step process. However, the nature of the local factors controlling marrow stromal cell differentiation and the interactions between them remains unidentified.

Transforming growth factor-β (TGF-β) and the TGF-β-related bone morphogenetic proteins (BMPs) are believed to play a major role in the control of bone formation [Urist, 1965;

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Wozney, 1992; Centrella et al., 1994; Linkhart et al., 1996; Marie, 1997]. TGF-β increases bone formation in vivo [Noda and Camillière, 1989; Machwate et al., 1995], and induces multiple cellular effects on osteoblast activity in vitro. TGF-β can either stimulate or inhibit cell proliferation and alkaline phosphatase activity [Centrella et al., 1987; Gehron-Robey et al., 1987; Lomri and Marie, 1990; Ingram et al, 1994], depending on the cell maturation stage. In addition, TGF-β is an important regulator of the synthesis and deposition of extracellular matrix components [Ignotz and Massagué, 1986; Centrella et al., 1987; Pfeilschifter et al., 1990]. TGF-β (TGF-β_1 or TGF-β_2) were recently shown to influence human bone marrow stromal cells. However, these factors do not induce complete osteogenesis in vitro in human bone marrow stromal cell cultures [Long et al., 1995; Fromigué et al, 1997].

Among members of the TGF-β superfamilly, the subfamilly of BMPs is composed of at least 15 molecules [Celeste et al., 1990; Kingsley et al., 1994]. Several BMPs have been cloned and expressed as recombinant proteins [Wozney et al., 1988]. BMP molecules are able to induce bone formation in vivo and to promote osteoblast differentiation in vitro [Katagiri et al., 1990; Wang et al., 1990; Chen et al., 1991; Sampath et al., 1992; Thies et al., 1992; Rickard et al., 1994]. BMP-3 (osteogenin) was reported to increase ALP activity, Col I synthesis and OC production in human bone marrow stromal cells [Amédée et al., 1994]. BMP-2 also increases the expression of osteoblastic markers such as ALP activity, OC and osteopontin, in rat bone marrow stromal cells [Rickard et al., 1994], and in mouse W20-17 marrow stromal cells [Thies et al., 1992]. However, the effects of BMP-2 on human marrow stromal cells are unknown.

We hypothesized that TGF-β and BMP-2 may interact at different stages to regulate HBMS cell differentiation in a coordinate manner. We therefore compared the in vitro effects of BMP-2 and TGF-β_2 on human bone marrow stromal cell growth and differentiation toward the osteoblastic lineage. In addition, we studied the interactions of these factors in short-term and long-term cultures. Our results show that rhTGF-β_2 stimulates cell growth whereas rhBMP-2 promotes the expression of markers of osteoblast differentiation. Furthermore, these two molecules interact with each other at distinct stages to modulate the proliferation and differentiation of human bone marrow stromal cells.

MATERIALS AND METHODS

Materials

Multiwell plates and culture dishes were purchased from Falcon (Becton-Dickinson-France, Le Pont de Claix, France). Dexamethasone, bovine serum albumin (BSA), Hoechst 33258 reagent, Naphthol AS-BI phosphate, and Fast Red Violet LB salt were from Sigma Chemical Co (Saint Louis, MO). Dulbecco's Modified Eagle Medium (DMEM), penicillin (10,000 UI/ml), streptomycin (10,000 µg/ml), trypsin-EDTA, and Extract-all reagent were from Eurobio (Les Ulis, France). Alkaline phosphatase and calcium detection kits were purchased from bioMerieux (Lyon, France). BioRad reagent was from Bio-Rad Laboratories (Ivry sur Seine, France). Procollagen type I C-terminal propeptide (P1CP) and osteocalcin radioimmunoassay detection kits were from Orion Diagnostica (Finland) and CIS Bio- international (Gif/Yvette, France), respectively. [γ^{32}P]ATP was purchased from NEN (Du Pont de Nemours, Les Ulis, France). Hybond-N+ membranes were from Appligene (Illkirch, France). Recombinant human bone morphogenetic protein 2 (rhBMP-2) was generously provided by Genetics Institute (Cambridge, MA). The stock solution (2.27 µg/µl) was aliquoted, and stored at -80°C. Recombinant human transforming growth factor-β_2 (rhTGF-β_2) was kindly provided by Novartis (Basel, Switzerland). Lyophilized rhTGF-β_2 was dissolved to a concentration of 50 ng/µl in 10 mM HCl with 10% ethanol and 1 mg/ml bovine serum albumin (BSA), then aliquoted, and stored at -80°C as stock solution.

Human Bone Marrow Cell Cultures

Human bone marrow stromal (HBMS) cells were obtained as described previously [Fromigué et al., 1997]. Normal human trabecular bone fragments obtained from the femoral neck of 14 patients (mean age = 68 years) undergoing local surgery were washed in phosphate-buffered salt (PBS), and bone marrow cells were collected by centrifugation at 1,200 rpm for 10 min. Hematopoietic cells were lysed by addition of NH_4Cl (0.17 M) for 10 min on ice, and the reaction was stopped by addition of DMEM followed by centrifugation at 1,200 rpm for 10 min. Marrow stromal cells were then

transferred to a 100-mm^2 dish (5×10^6 cells/cm^2) containing DMEM with 10% fetal calf serum (FCS), 50 mg/ml ascorbic acid, 3 mM NaH_2PO_4, and 10^{-8} M dexamethasone, and allowed to adhere for 48 h in a humidified atmosphere of 5% CO_2–95% air at 37°C. After 48 h, nonadherent cells were removed by three washes in serum-free medium and residual adherent cells were cultured in the presence of 10% FCS. At confluence (2–3 weeks), HBMS cells were removed with a trypsin/EDTA solution, collected and seeded at 12,500 cells/cm^2 in 24-well plates for biochemical analysis and histochemical staining, and in 100-mm^2 dishes for gene expression analysis.

The effects of rhBMP-2 and rhTGF-β_2 on HBMS cells were studied in short-term and long-term cultures. For short-term studies, confluent HBMS cells were cultured in DMEM supplemented with 1% FCS and 0.1% BSA for 48 h. Treatment with rhBMP-2 or/and rhTGF-β_2 was then applied in the same medium supplemented with 50 µg/ml ascorbic acid for 7 days, with media changed every 2 days. For long-term cultures, confluent HBMS cells were treated for 21 days with rhBMP-2 and rhTGF-β_2, alone or in combination, in DMEM supplemented with 10% FCS to allow optimal cell metabolism in long-term cultures, 50 µg/ml ascorbic acid, and 3 mM phosphate, with media changed every 2 days. We also performed sequential treatments in long-term cultures. Confluent HBMS cells were treated sequentially either with rhBMP-2 from day 0 to day 9, followed by rhTGF-β_2 from day 9 to day 19, or with rhTGF-β_2 from day 0 to day 10, followed by rhBMP-2 from day 10 to day 21. DNA content, ALP activity, osteocalcin and P1CP levels and calcium content were measured at timed-intervals, as described below.

Analysis of DNA Content

Total DNA content was determined by a fluorometric procedure. After medium removal, HBMS cells were washed in PBS, lysed in cold bidistilled water, scraped, and sonicated on ice. An aliquot of each cell lysate was lyophilized, dissolved in 10 mM EDTA (pH 12.3), neutralized by addition of KH_2PO_4 (1 M), and incubated with Hoechst 33258 reagent [West et al., 1985]. Fluorescence was measured at excitation and emission wavelengths of 350 nm and 455 nm, respectively. Calf thymus DNA was used for standardisation. The results are expressed as micrograms of DNA per well or as a ratio of treated over control.

Alkaline Phosphatase Activity

Alkaline phosphatase activity was evaluated by a colorimetric procedure and the results expressed as nanomoles p-nitrophenol released per min/mg protein or as a ratio of treated over control. Total protein content was determined biochemically using the BioRad Protein Assay. A standard curve was prepared using BSA. ALP activity was also identified histochemically on HBMS cells washed in PBS and fixed in 70% ethanol at 4°C for 30 min. HBMS cells were then incubated at 37°C for 1 h in Tris buffer (pH 8.5) containing naphthol AS-BI phosphate and Fast Red Violet LB-salt.

Type I Collagen Synthesis

Conditioned media, removed at several time points, were aliquoted and stored at -20°C. Type I collagen synthesis was estimated by determination of procollagen type I C-terminal propeptide concentrations in the conditioned media, using a procollagen [^{125}I] RIA kit [Fromigué et al., 1997]. The intra- and interassay variability are 3.2% and 4.0%, respectively, for the range of concentrations evaluated. Results are expressed as µg P1CP/mg protein or as a ratio of treated over control.

Osteocalcin Production

Osteocalcin levels were evaluated on conditioned media at several time-points using a specific anti-bovine osteocalcin antibody [Fromigué et al., 1997]. The detection limit is 0.35 ng/ml, and the intra- and interassay variability are 3.7% and 6.6%, respectively, for the range of concentrations evaluated. Values were expressed as percent of control after correction for protein content.

Calcium Content

To evaluate the effects of rhBMP-2 and rhTGF-β_2 on the mineralizing potential of HBMS cells in long-term culture, total calcium content in the extracellular matrix was determined throughout the 3-week period. At timed intervals, cell layers were washed three times with PBS and sonicated in H_2O. Aliquots were used for DNA content determination. Formic acid was added to the remaining cell lysate to a final concentration of 10%. A biochemical deter-

TABLE I. Sequence of Specific Oligonucleotide Primers Used for PCR Amplification and Hybridization

	Primer sequence	Fragment length of PCR products (bp)
ALP		
Sense	5′-CCCAAAGGCTTCTTCTTG-3′	357
Antisense	5′-CTGGTAGTTGTTGTGAGC-3′	
Internal	5′-GTCTGTGTCACTCAGCATGG-3′	
Col I		
Sense	5′-GGACACAATGGATTGCAAGG-3′	461
Antisense	5′-TAACCACTGCTCCACTCTGG-3′	
Internal	5′-TAACCACCACCGCTTACACC-3′	
GAPDH		
Sense	5′-GGGCTGCTTTTAACTCTGGT-3′	702
Antisense	5′-TGGCAGGTTTTTCTAGACGG-3′	
Internal	5′-CATGAGTCCTTCCACGATACC-3′	

mination of total calcium content (μg/well) was performed by a colorimetric assay using a calcium detection kit, and the results expressed as the ratio treated over control. Mineral deposition in the matrix was also identified histochemically by von Kossa staining.

Analysis of Gene Expression

The expression of alkaline phosphatase, and type I collagen were analyzed by reverse transcription-polymerase chain reaction, as previously described [Fromigué et al., 1997]. After 5 days of culture in the presence of rhBMP-2 or rhTGF-β_2, alone or in combination, HBMS cells were washed in PBS and lysed in Extract-All reagent according to the manufacturer's protocol. Two μg total RNA from each samples were reverse transcribed using MMLV reverse transcriptase (200 units) and randomized hexanucleotides (100 pmol) as primers. The reverse transcription was performed at 37°C for 1 h and stopped by heating 10 min at 85°C. cDNA samples were then divided and amplified using specific primers for ALP, type I collagen and GAPDH (Table I). After 3-min denaturation at 95°C, PCR was performed for 30 cycles consisting of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, followed by a final extension step of 6 min at 72°C before cooling at 4°C. 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 (Table I). Membranes were washed twice in 2× SSC/0.1% SDS at room temperature for 10 min, then in 0.1× SSC/0.1% SDS at 50°C for 10 min, and filters were exposed to X-ray intensifying screen. Autoradiographic signals were quantified using a scanning densitometer and corrected for GAPDH expression (Transydine General Corporation, Ann Arbor, MI).

Statistical Analysis

All experiments were performed in at least two different batches from separate donors. The data presented are representative of the results obtained in the diferent cell batches. Data are expressed as the mean ±SEM of 3–4 independant cultures. Statistical analysis was performed using the statistical package super ANOVA (Macintosh, Abacus Concepts Inc., Berkeley, CA) with a minimal significance of $P < 0.05$.

RESULTS

Dose-Response Effects of rhBMP-2 and rhTGF-β_2 on HBMS Cells

We first analyzed the dose-response effects of rhBMP-2 and rhTGF-β_2 on human bone marrow stromal cells treated for 5 days with increasing concentrations of rhBMP-2 and rhTGF-β_2. The addition of rhBMP-2 (50–100 ng/ml) to HBMS cell cultures led to a dose-dependent decrease in total DNA content with a maximal effect (-35%) at 100 ng/ml (Fig. 1A). In contrast, treatment with rhTGF-β_2 induced an increase in HBMS cell proliferation, with a maximal effect (+25%) at 1–5 ng/ml (Fig. 1A). As shown in Figure 1B, treatment with rhBMP-2 stimulated ALP activity in a dose-dependent manner. The effect was detectable at 50–100 ng/ml with

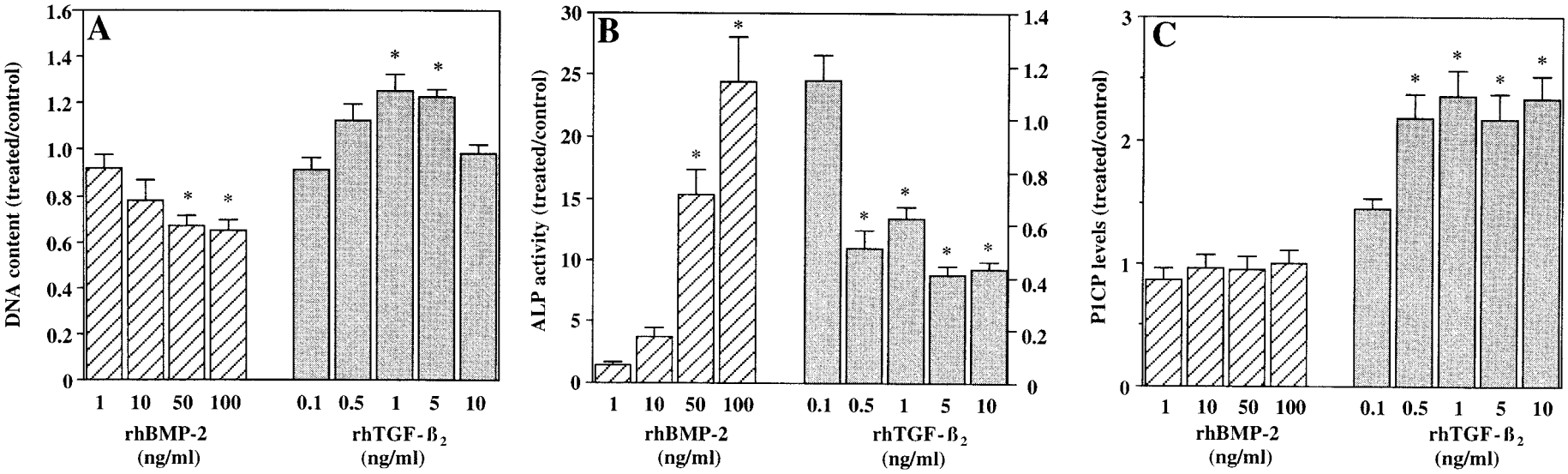

Fig. 1. Dose-response effects of rhBMP-2 and rhTGF-β_2 on total DNA content **(A)**, alkaline phosphatase activity **(B)**, and type I collagen synthesis, evaluated by P1CP levels **(C)** in HBMS cells. Confluent HBMS cells cultured for 48 h in the presence of 1% FCS prior to treatment, were treated with increasing concentrations of rhBMP-2 and rhTGF-β_2 for 5 days. Data are the means +SEM of 3-4 independent wells. *: $P < 0.05$ compared to untreated cells.

a 15.3- to 24.5-fold increase compared to the basal level. In contrast, ALP activity was inhibited by about 50% at low dosage levels of rhTGF-β_2 (Fig. 1B). In parallel, rhTGF-β_2 increased type I collagen synthesis as evaluated by P1CP levels in the medium, whereas rhBMP-2 had no significant effect (Fig. 1C). The stimulatory effect of rhTGF-β_2 on type I collagen synthesis was found at 0.5–10 ng/ml (2.2- to 2.5-fold, Fig. 1C). These results show that rhTGF-β_2 and rhBMP-2 have divergent effects on HBMS cell growth and osteoblastic markers. The effective doses of 100 ng/ml of rhBMP-2 and 1 ng/ml of rhTGF-β_2 were thereafter chosen for the following studies on HBMS cell proliferation and differentiation.

rhBMP-2 and rhTGF-β_2 Have Opposite Effects on HBMS Cells

We then evaluated the effects of rhBMP-2 and rhTGF-β_2 on HBMS cells by investigating the changes in the expression of several osteoblastic markers at different time points during a short-term culture (0–7 days). In basal conditions, HBMS cells exhibited low ALP activity. ALP activity decreased by 40% between day 0 and day 7 in control cells (Fig. 2A). Treatment with rhTGF-β_2 further reduced ALP activity by 50% at 5–7 days. In contrast, treatment of HBMS cells with rhBMP-2 led to a marked increase (3.5-fold) in ALP activity at the same period of time. When HBMS cells were treated with rhBMP-2 and rhTGF-β_2, an intermediate effect was obtained, as rhTGF-β_2 markedly attenuated the stimulatory effect of rhBMP-2 (Fig. 2A). These results show that rhBMP-2 and rhTGF-β_2 have opposite effects on ALP activity at early stages of the culture.

Type I collagen synthesis in HBMS cell cultures was evaluated by determination of P1CP levels in conditioned media. In basal conditions, P1CP levels increased markedly (10-fold) and reached a maximum value at day 5 (Fig. 2B). Treatment with rhTGF-β_2 increased by about 80% P1CP levels at 5–7 days. In contrast, rhBMP-2 did not affect P1CP levels. In addition, rhBMP-2 combined with rhTGF-β_2 reduced the stimulatory effect of rhTGF-β_2 on P1CP levels. These data indicate that rhTGF-β_2 and rhBMP-2 have opposite effects on type I collagen synthesis in short-term culture.

To evaluate whether rhTGF-β_2 and rhBMP-2 controlled ALP activity and type I collagen in the same manner at the gene level, the expression of ALP and type I collagen was analyzed by RT-PCR after 5 days of culture. Figure 3 shows autoradiograms of the hybridized membranes and the relative expression of ALP and type I collagen after correction for GAPDH levels. At this time point, osteocalcin mRNA were not detectable in HBMS cells (not shown). We found that rhBMP-2 increased ALP mRNA levels by

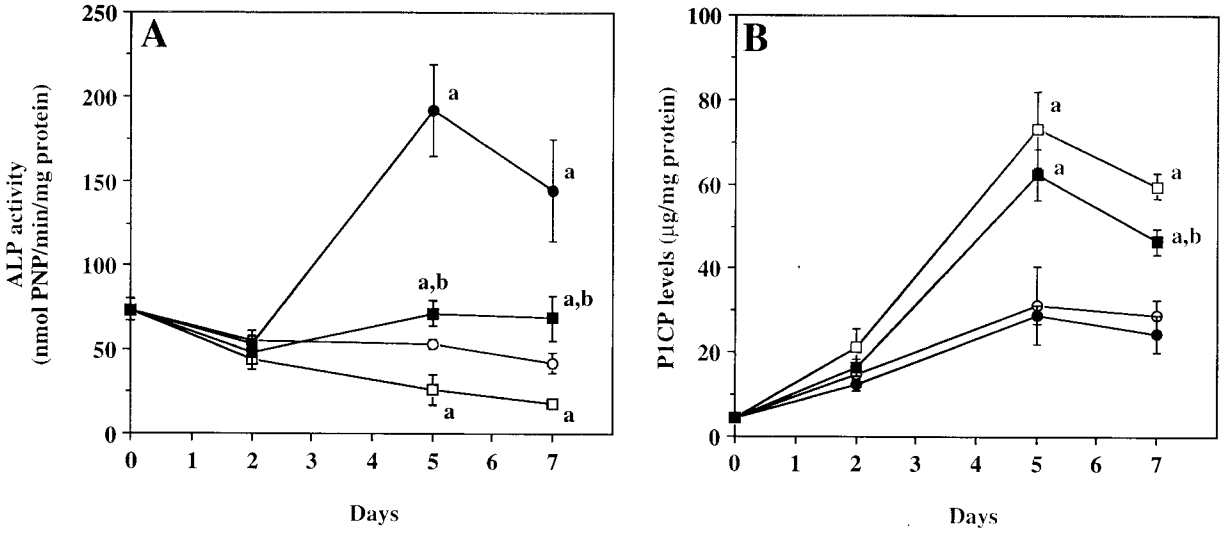

Fig. 2. Effects of rhBMP-2 (●, 100 ng/ml), rhTGF-β_2 (□, 1 ng/ml), and their combination (■) on alkaline phosphatase activity **(A)**, and type I collagen synthesis evaluated as P1CP levels **(B)** in HBMS cells in short-term culture, compared to untreated cells (o, control). HBMS cells were grown to confluence, then placed in DMEM supplemented with 1% FCS for 48 h prior to treatment. Data are the means ±SEM of 3–4 independent wells. **a:** $P < 0.05$ compared to untreated cells. **b**: $P < 0.05$ vs rhBMP-2- and rhTGF-β_2-treated cells.

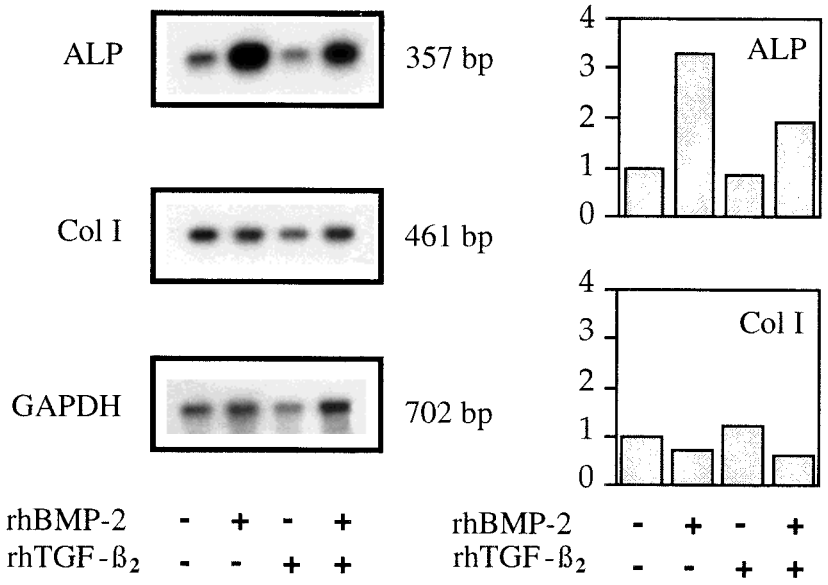

Fig. 3. Effects of rhBMP-2 (100 ng/ml) and rhTGF-β_2 (1 ng/ml) on the expression of alkaline phosphatase (ALP) and type I collagen (Col I) mRNA levels in confluent HBMS cell cultures. Total RNA was extracted after 5 days exposure to rhBMP-2, rhTGF-β_2 or the two factors, and ALP expression was evaluated by RT-PCR analysis. **A:** Autoradiograms of amplified cDNA hybridized to [γ^{32}P]-labeled probes encoding for ALP, Col I, and GAPDH. **B**: Relative expression of ALP and Col I mRNA corrected for the amounts of mRNA loaded using GAPDH mRNA.

3.3-fold, whereas rhTGF-β_2 reduced ALP transcripts. When both rhBMP-2 and rhTGF-β_2 were applied on HBMS cells, an intermediate effect on ALP transcripts was observed. These effects were confirmed using Northern blot analysis (data not shown). HBMS cells expressed low levels of type I collagen transcripts in control as well as in treated cultures. Type I collagen mRNA levels, estimated by RT-PCR, were not markedly modified by treatments (Fig. 3). These data indicate that rhTGF-β_2 and rhBMP-2 have opposite effects on ALP mRNA, but not on type I collagen mRNA levels, in these culture conditions.

rhBMP-2 and rhTGF-β_2 Have Opposite Effects on Differentiating HBMS Cells

The effects of rhBMP-2 and rhTGF-β_2 on the proliferation and differentiation of HBMS cells were then analyzed during a 3-week period, when HBMS cells were induced to differentiate and formed a mineralized matrix in the presence of ascorbic acid and phosphate. Figure 4A shows the effects of rhBMP-2 and rhTGF-β_2, alone or in combination, on total DNA content in long-term HBMS cell cultures. Treatment with rhBMP-2 reduced by 40% HBMS cell growth, as shown by DNA content at 2–3 weeks of culture, compared to untreated cells. In contrast, rhTGF-β_2 transiently stimulated DNA synthesis by 60% at 2 weeks of culture. The combination of rhBMP-2 and rhTGF-β_2 had no effect on DNA content compared to control cells, showing that the stimulatory effect of rhTGF-β_2 was suppressed by the addition of rhBMP-2.

Figure 4B shows that, under basal culture conditions, ALP activity decreased progressively by about 6-fold between day 0 and day 21 of culture. Treatment with rhBMP-2 induced a marked increase in ALP activity at 2–21 days of culture, with a maximal effect (5-fold) at 7 days. In contrast, treatment with rhTGF-β_2 reduced ALP activity by 50% at 7–21 days. Treatment with rhBMP-2 associated with rhTGF-β_2 led to an intermediate effect on ALP activity in HBMS cells (Fig. 4B). These biochemical results were confirmed by histochemical staining. Figure 5 illustrates the changes in ALP-positive cells (shown in gray) and matrix mineralization shown by von Kossa staining (shown in black), at 21 days of culture. rhBMP-2 increased ALP staining in HBMS cells (Fig. 5b), compared to untreated cells (Fig. 5a). In contrast, rhTGF-β_2 reduced the number of ALP-positive cells (Fig. 5c), and rhTGF-β_2 inhibited the stimulatory effect of rhBMP-2 on ALP activity in HBMS cells (Fig. 5d). These results show that rhBMP-2 and rhTGF-β_2 have opposite effects on

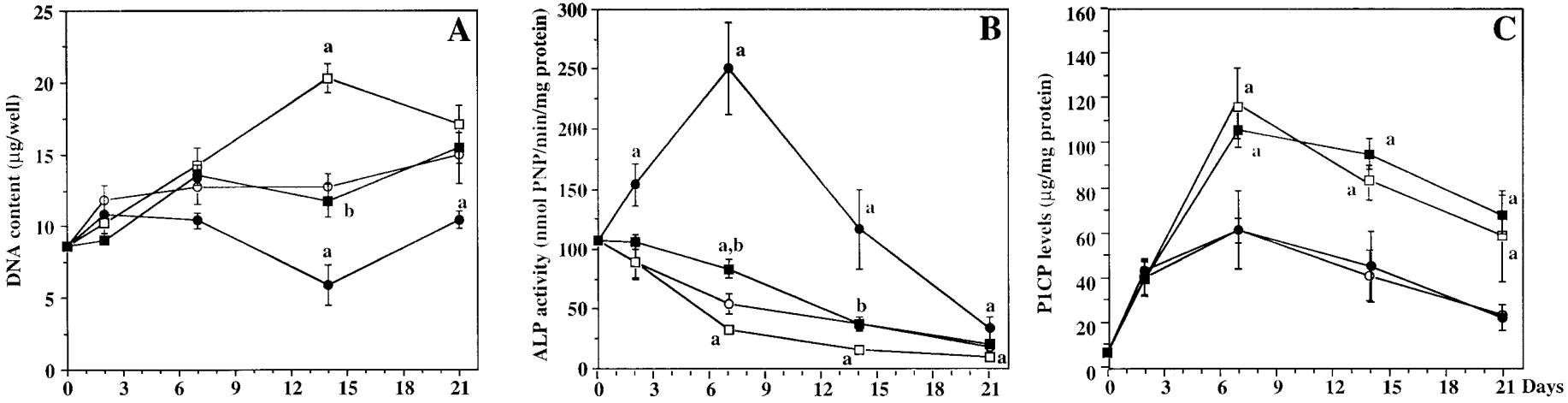

Fig. 4. Effect of long-term treatment with rhBMP-2 (●, 100 ng/ml), rhTGF-β_2 (□, 1 ng/ml), or their combination (■) on total DNA content (**A**), ALP activity (**B**), and P1CP levels (**C**) in differentiating HBMS cells, compared to untreated cells (○, control). Confluent cells were cultured in DMEM supplemented with 10% FCS, 50 µg/ml ascorbic acid, and 3 mM phosphate, in the presence of rhBMP-2 and/or rhTGF-β_2. Data represent means ±SEM of 3–4 independent wells. **a**: $P < 0.05$ compared to untreated cells. **b**: $P < 0.05$ vs rhBMP-2- and rhTGF-β_2-treated cells.

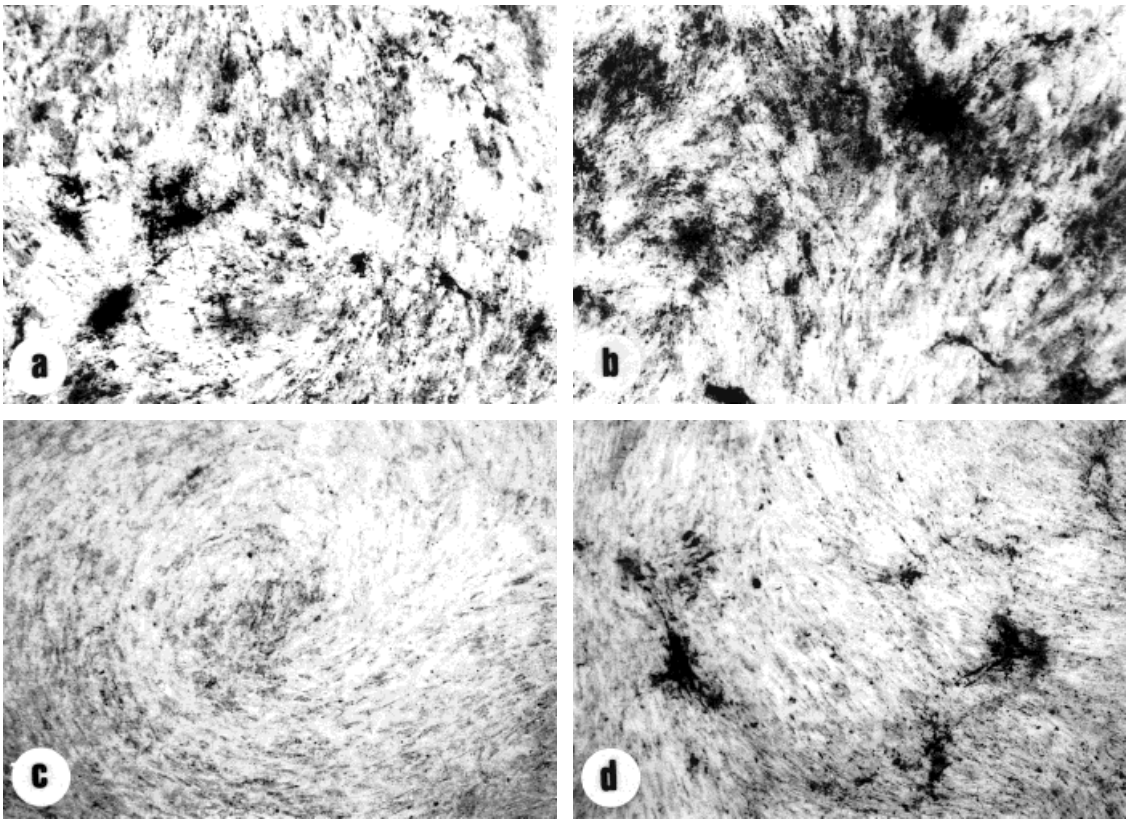

Fig. 5. Effects of rhBMP-2 (**b**, 100 ng/ml) and rhTGF-β_2 (**c**, 1 ng/ml) and their combination (**d**) compared to untreated cells (**a**) on ALP activity and mineral deposition evaluated histochemically. HBMS cells were cultured for 21 days in the presence of ascorbic acid and phosphate, then fixed in 75% ethanol and stained for ALP and von Kossa. ALP-positive HBMS cells appeared in gray and calcium deposition appears in black. Magnification: ×25.

the number of cells expressing ALP activity in HBMS cells induced to differentiate in long-term culture.

Table II shows the effects of rhTGF-β_2 and rhBMP-2 on osteocalcin production. Osteocalcin levels were not detectable in the medium before 1 week of culture. We found that osteocalcin levels were decreased by rhTGF-β_2 by about 40% at 2 weeks of culture (Table II). A similar effect was observed at 1–4 weeks (-40%, $P < 0.05$). In contrast, rhBMP-2 markedly increased osteocalcin levels at 2 weeks (Table II), as well as at 3–4 weeks (3-fold, $P < 0.05$). rhTGF-β_2 reduced the stimulatory effect of rhBMP-2 on osteocalcin production (Table II), showing that rhTGF-β_2 and rhBMP-2 had opposite effects on osteocalcin production by HBMS cells.

Figure 4C shows that P1CP levels in basal conditions increased with time with a maximum level at day 7, and then declined progressively with time. Treatment with rhTGF-β_2 increased P1CP levels (2-fold) compared to untreated cells, at 1–3 weeks. Treatment with rhBMP-2 did not significantly affect type I collagen synthesis, and the addition of rhBMP-2 to rhTGF-β_2 did not modify the stimulatory effect of rhTGF-β_2. Thus, rhBMP-2 and rhTGF-β_2 had differential effects on type I collagen production by HBMS cells in long-term culture.

In basal culture conditions, in the presence of inorganic phosphate, mineralization of the ma-

TABLE II. Effects of rhBMP-2 (100 ng/ml) and rhTGF-β_2 (1 ng/ml) on Osteocalcin Production by Human Bone Marrow Stromal Cells at 2 Weeks of Culture[†]

	Osteocalcin (% control)
Control	100 ± 14
rhBMP-2	377 ± 48*
rhTGF-β2	53 ± 5*
rhBMP-2 + rhTGF-β_2	151 ± 15*,**

[†]Data represent the mean ± SEM of three values.
*Significant difference with control ($P < 0.02$).
**Significant difference with rhBMP-2 and rhTGF-β_2 ($P < 0.05$).

trix increased progressively with time as assessed by total calcium content, starting at day 7 (7.12 + 0.20 µg/ µg DNA vs 3.87 + 0.31 µg/µg DNA at day 0) with a maximal value at day 21 (8.77 + 0.59 µg/µg DNA). Treatment with rhBMP-2 increased calcium deposition by 67% ($P < 0.02$) at 14 days of culture. In contrast, treatment with rhTGF-β_2 decreased calcium content at 7-14 days of culture (-20%, $P < 0.05$). This was confirmed by the histochemical analysis of mineral deposition in the matrix (Fig. 5), which also shows that the combination of treatments led to an intermediate effect on matrix mineralization, appearing in black by von Kossa staining (Fig. 5d). These results show that rhBMP-2 and rhTGF-β_2 have opposite effects on the mineralization of the extracellular matrix produced by HBMS cells in long-term culture.

Transient Treatment With rhBMP-2 Partly Suppresses rhTGF-β_2 Effects on HBMS Cells

To determine the stage at which rhBMP-2 and rhTGF-β_2 modulate the effects of each other, HBMS cells were submitted to a sequential treatment in long-term culture. During the first 9 days of culture, rhBMP-2 was applied alone, and then the treatment was switched to rhTGF-β_2 for a further period of 10 days. Figure 6A shows that transient treatment with rhBMP-2 for 9 days had no effect on DNA synthesis, and this treatment did not modify the subsequent action of rhTGF-β_2 on DNA synthesis. Indeed, when rhTGF-β_2 was applied after rhBMP-2 treatment the expected stimulatory effect of rhTGF-β_2 on DNA synthesis (Fig. 4A) was observed. These data suggest that a transient treatment with rhBMP-2 does not affect HBMS cell responsiveness to the mitogenic effect of rhTGF-β_2.

We then studied the effect of this sequential treatment on markers of osteoblast differentiation. Figure 6B shows that a transient treatment with rhBMP-2 increased ALP activity with a maximal effect at day 5 (4.1-fold) as previously observed (Fig. 4B). When rhBMP-2 was replaced with rhTGF-β_2 at day 9, ALP activity returned to basal levels. However, the subsequent treatment with rhTGF-β_2 did not reduce ALP activity to levels below those of untreated cells, indicating that the transient treatment with rhBMP-2 abolished the response of HBMS cells to rhTGF-β_2 in terms of ALP activity. As previously observed (Fig. 4C), the transient treatment with rhBMP-2 during 9 days did not affect type I collagen synthesis evaluated by P1CP levels. After the switch to rhTGF-β_2, type I collagen synthesis increased by about 60-80% from 14 to 19 days of treatment compared to untreated cells ($P < 0.01$), showing that the transient early treatment with rhBMP-2 did not affect the stimulatory effect of rhTGF-β_2 on type I collagen synthesis in HBMS cells. Finally, we studied the effects of this sequential treatment on matrix mineralization (Fig. 6C). Despite the transient treatment with rhBMP-2, the following treatment with rhTGF-β_2 decreased calcium content in the matrix (-35–50%), showing that the transient treatment with rhBMP-2 did not affect the inhibitory effect of rhTGF-β_2 on matrix mineralization. Taken together, these results show that the sequential treatment with rhBMP-2 affects partially the response of HBMS cells to rhTGF-β_2, since rhBMP-2 abolished the effects of rhTGF-β_2 on ALP activity, but not on DNA synthesis, type I collagen, or matrix mineralization.

Transient Treatment With rhTGF-β_2 Does Not Oppose rhBMP-2 Effects on HBMS Cells

To evaluate whether rhTGF-β_2 affects the response of HBMS cells to rhBMP-2, the inverse sequential combination of treatment with the two factors was tested in long-term culture. HBMS cells were treated with rhTGF-β_2 from day 0 to day 10, then with rhBMP-2 from day 10 to day 21. Figure 7A shows that the early treatment with rhTGF-β_2 increased cell proliferation and that the switch to rhBMP-2 abolished this effect, showing that the transient treatment with rhTGF-β_2 did not influence the response to rhBMP-2. As found with the long-term continuous treatment (Fig. 4B), the transient treatment with rhTGF-β_2 inibited ALP activity (-56% at 7 days, Fig. 7B). When rhTGF-β_2 was switched to rhBMP-2, ALP activity was markedly increased (6.1-fold at 21 days; Fig. 7B), indicating that the transient treatment with rhTGF-β_2 did not affect the response to rhBMP-2 in terms of ALP activity. The transient treatment with rhTGF-β_2 increased type I collagen synthesis (2-fold at day 7, $P < 0.05$) as observed previously (Fig. 4C). When rhTGF-β_2 was changed to rhBMP-2, P1CP levels returned to basal levels (data not shown), as expected from the observed lack of effect of rhBMP-2 on P1CP levels (Fig. 4C). The transient treatment with rhTGF-β_2 had a potent inhibitory effect on

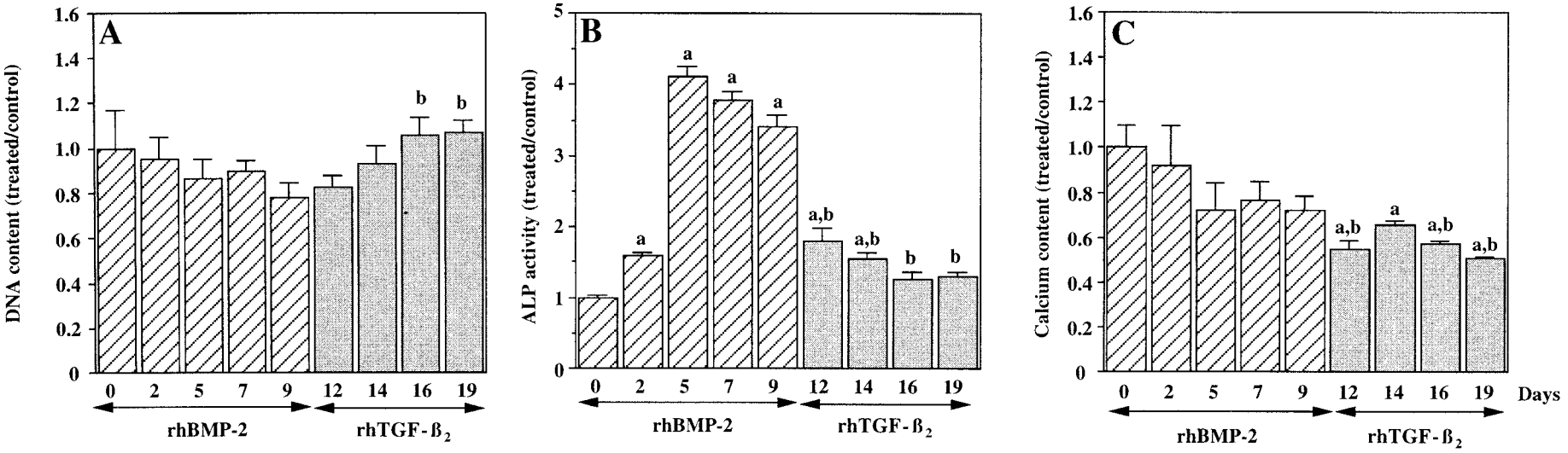

Fig. 6. Effect of a sequential treatment with rhBMP-2 (100 ng/ml) and rhTGF-β_2 (1 ng/ml) on HBMS cells. Confluent cell cultures were treated with rhBMP-2 alone for 9 days, then with rhTGF-β_2 alone for further 10 days, in the same medium as for the long-term treatment. Total DNA content **(A)**, ALP activity **(B)**, and calcium content **(C)** were determined throughout the culture. Data represent the means ±SEM of 3–4 independent wells and expressed as treated/control. **a**: $P < 0.05$ compared to day 0, **b**: $P < 0.05$ compared to day 9.

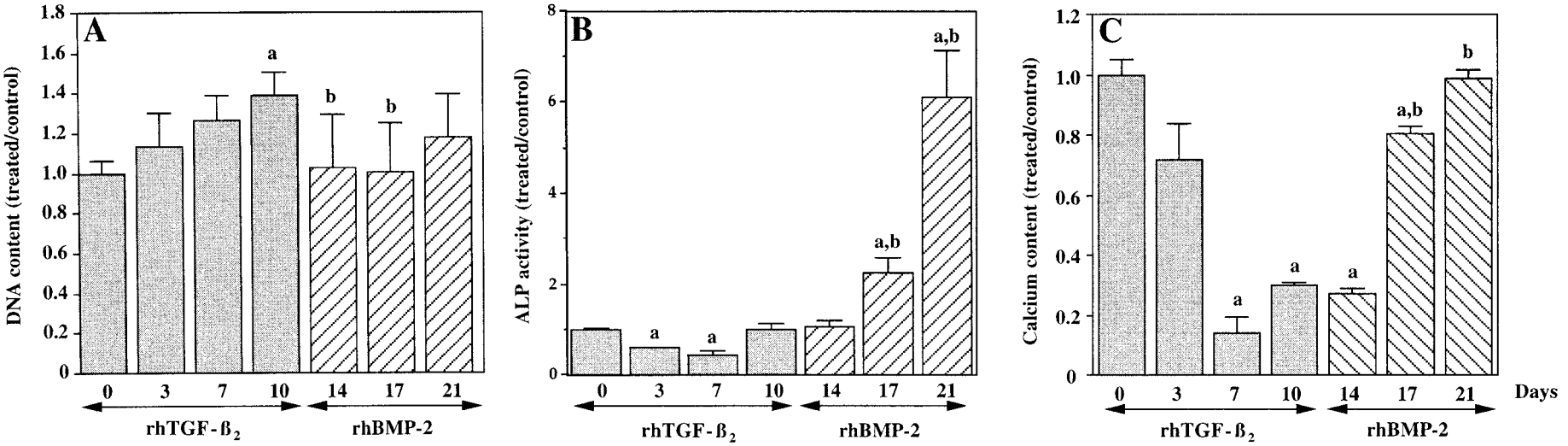

Fig. 7. Effect of a sequential treatment with rhBMP-2 (100 ng/ml), and rhTGF-β_2 (1 ng/ml) on HBMS cells. Confluent cell cultures were treated with rhTGF-β_2 alone for 10 days, then with rhBMP-2 alone for further 11 days, in the same medium as for the long-term treatment. Total DNA content **(A)**, ALP activity **(B)**, and calcium content **(C)** were determined throughout the culture. Data represent the means ±SEM of 3–4 independent wells and expressed as treated/control. **a**: $P < 0.05$ compared to day 0, **b**: $P < 0.05$ compared to day 10.

TABLE III. Interactions of rhTGF-β_2 and rhBMP-2 on Human Bone Marrow Stromal Cell Proliferation and Differentiation*

	DNA	ALP	Type I collagen	Matrix mineralization
Continuous treatments				
rhBMP-2 alone (21 days)	−	++	0	+
rhTGF-β_2 alone (21 days)	+	−	++	−
rhBMP-2 + rhTGF-β_2 (21 days)	0	+	+	0
Sequential treatments				
rhBMP-2 (9 days)	0	++	0	0
then rhTGF-β_2 (10 days)	+	0	+	−
rhTGF-β_2 (10 days)	+	−	+	−
then rhBMP-2 (11 days)	0	++	0	+

*rhTGF-β_2 (1 ng/ml) and rhBMP-2 (100 ng/ml) had opposite effects and the combined treatment induced intermediate effects on HBMS cell proliferation and differentiation.
−, +, ++, decrease, an increase, or a marked increase compared to untreated cells; 0, no change or an intermediate effect compared to untreated cells.

calcium deposition in the matrix (-86% at day 7, Fig. 7C). After day 10, when rhTGF-β_2 was switched to rhBMP-2, calcium content increased and returned to basal level at day 21, showing that the transient treatment with rhTGF-β_2 did not affect the stimulatory effect of rhBMP-2 on matrix mineralization. These data indicate that the early treatment with rhTGF-β_2, which increased HBMS cell growth and type I collagen synthesis, reduced ALP activity and matrix mineralization, and that the following treatment with rhBMP-2 reversed completely the rhTGF-β_2 effects, and also enhanced markers of the osteoblastic phenotype in HBMS cell cultures.

The effects and interactions of rhTGF-β_2 and rhBMP-2 on HBMS cell proliferation and differentiation are summarized in Table III. Taken together, the data show that (1) rhTGF-β_2 and rhBMP-2 have opposite effects on human bone marrow stromal cell proliferation and differentiation markers, and each factor modulates the action of the other; (2) the early treatment with rhBMP-2 abolishes in part the cellular response of HBMS cells to rhTGF-β_2, whereas the early treatment with rhTGF-β_2 does not modify the HBMS cell response to rhBMP-2; and (3) rhTGF-β_2 acts on HBMS cell proliferation whereas rhBMP-2 promotes HBMS cell differentiation towards the osteoblastic lineage.

DISCUSSION

Although several local factors may control the commitment and differentiation of osteoblast precursor cells present in the bone marrow stroma, the nature of these factors and the interactions between them remain to be determined. In the present study, we show that rhBMP-2 and rhTGF-β_2 have differential effects on human bone marrow stromal cells, and that the two factors modulate the action of each other and act at distinct stages to promote HBMS cell proliferation and differentiation.

Analysis of DNA synthesis showed that rhTGF-β_2 and rhBMP-2 had opposite effects on HBMS cell growth. Indeed, rhBMP-2 decreased DNA content, whereas rhTGF-β_2 increased it in short-term as well as in long-term cultures. Variable effects of TGF on osteoblastic cell growth have been described, depending on the cell type or culture conditions [Centrella et al., 1987; Lomri and Marie, 1990; Chen et al., 1991; Harris et al., 1994; Machwate et al., 1995; Fromigué et al., 1997]. The effects of other members of the TGF-β family are also concentration-dependent in several systems [Chen et al., 1991; Hiraki et al., 1991; Dieudonné et al., 1994; Hughes et al., 1995; Yamaguchi et al., 1996]. Low doses of BMP-3 (osteogenin), which shares about 60% identity with BMP-2, were found to have a strong inhibitory effect on human bone marrow stromal cell proliferation [Amédée et al., 1994]. In contrast, we found that only high doses of rhBMP-2 decreased HBMS cell growth. Thus, BMP-2 does not appear to be an important modulator of HBMS cell growth compared to BMP-3 or rhTGF-β_2.

We found that rhBMP-2 and rhTGF-β_2 also had opposite effects on ALP activity, an early marker of the osteoblastic phenotype. Low concentrations of rhTGF-β_2 inhibited ALP activity, whereas rhBMP-2 had stimulatory effects. Moreover, even at concentration of BMP-2 as high as 100 ng/ml, the response had not pla-

teaued. This is consistent with data from other studies in W20-17 mouse stromal cells [Thies et al., 1992] indicating that the increase in ALP activity in response to BMP-2 did not plateau even at concentration as high as 800 ng/ml. Analysis at the gene level confirmed our data obtained at the protein level since ALP mRNA expression was increased by rhBMP-2 and decreased by rhTGF-β_2. The opposite effects of rhBMP-2 and rhTGF-β_2 on ALP activity indicate that rhBMP-2 may induce the commitment of precursor cells toward the osteoblastic lineage, whereas rhTGF-β_2 stimulates HBMS cell replication but does not promote their differentiation.

We found that rhTGF-β_2 and rhBMP-2 had distinct effects on collagen type I synthesis. rhTGF-β_2 greatly stimulated type I collagen synthesis in HBMS cells, which is consistent with the effect of TGF-β on the regulation of extracellular matrix protein production in osteoblastic cells [Centrella et al., 1987]. rhTGF-β_2 did not affect type I collagen mRNA levels, suggesting that rhTGF-β_2 acts at a post-transcriptional level in HBMS cells. Type I collagen synthesis is known to be regulated at both transcriptional and post-transcriptional levels in osteoblastic cells [Centrella et al., 1994]. In contrast, rhBMP-2 did not modify type I collagen synthesis and mRNA levels, even at high doses or in long-term treatment, and did not affect the marked stimulatory effect of rhTGF-β_2. The effect of BMPs on type I collagen was found to be variable in different systems. With regard to bone marrow stromal cells, BMP-2 or BMP-3 have been reported to induce a slight increase in type I collagen synthesis [Amédée et al., 1994; Rickard et al., 1994]. The lack of effect of rhBMP-2 on type I collagen synthesis in our model points to the different actions of rhBMP-2 and rhTGF-β_2 on human bone marrow stromal cells. This is also supported by our finding that rhTGF-β_2 decreased osteocalcin production, whereas rhBMP-2 increased osteocalcin levels in long-term HBMS cultures. Altogether, these data indicate that rhBMP-2 and rhTGF-β_2 influence the growth and differentiation of human bone marrow stromal cells, and have opposite effects on cell growth and markers of differentiation in short-term culture.

We also compared the effects of the two factors in long-term culture. When HBMS cells were induced to differentiate in the presence of ascorbic acid and phosphate, the cells initially proliferated, and type I collagen synthesis as well as ALP activity increased for up to 1 week. These markers decreased thereafter whereas calcium content in the extracellular matrix rose progressively [Fromigué et al., 1997]. In the present study, we found that rhBMP-2 decreased cell growth, increased ALP activity, and increased calcium content in the matrix independently of changes in type I collagen synthesis, indicating that rhBMP-2 stimulated HBMS cell differentiation towards the osteoblastic phenotype. The decreased cell proliferation induced by rhBMP-2 followed enhancement of ALP activity, indicating that this inhibition of cell growth was secondary to the induction of differentiation in HBMS cells. In contrast to the effect of rhBMP-2, we found that rhTGF-β_2 increased HBMS cell proliferation and type I collagen synthesis, and decreased ALP activity and matrix mineralization. The latter is in agreement with the inhibitory potential of TGFβ on the mineralization process observed in rat osteoblastic cells [Breen et al., 1994]. These results show that, in differentiating HBMS cells, rhBMP-2 and rhTGF-β_2 also exert opposite effects on cell proliferation, osteoblastic markers and matrix mineralization in long-term culture (Table III).

Since our data show that rhBMP-2 and rhTGF-β_2 distinctly modulate the proliferation and differentiation of human bone marrow stromal cells, we investigated the possibility that rhBMP-2 and rhTGF-β_2 may act in a coordinated manner on HBMS cell differentiation. We found that rhBMP-2 and rhTGF-β_2 attenuated the effect of each other when they were present throughout the long-term culture. Indeed, cotreatment conditions induced intermediate effects on HBMS cell proliferation and differentiation markers compared to treatment with each factor alone. rhTGF-β_2 blunted, in part, the stimulatory effect of rhBMP-2 on ALP activity and mRNA expression, which is consistent with the attenuation of BMP-2-enhanced ALP activity by TGFβ observed in mouse W20-17 stromal cells [Thies et al., 1992]. These data show that rhTGF-β_2 and rhBMP-2 interact to modulate cell proliferation and differentiation in human bone marrow stromal cells in long-term culture.

The reported effects of BMPs and TGFβ in rat bone marrow stromal cells [Hiraki et al., 1991] suggested that BMPs may influence the initial differentiation of mesenchymal cells

whereas TGFβ may stimulate the function of mature osteoblasts [Chen et al., 1991]. It is also possible that TGFβ may stimulate the recruitment and proliferation of osteoblast precursors, whereas BMPs may induce the differentiation of progenitor cells into mature osteoblasts [Bonewald and Dallas, 1994]. In order to investigate these two hypotheses, we tested whether sequential treatments with BMP-2 and TGF-β_2 may optimally promote HBMS cell differentiation and matrix mineralization. We found that a transient treatment with rhTGF-β_2 did not affect the effects of rhBMP-2 on HBMS cell differentiation. In contrast, transient treatment with rhBMP-2 during the first part of the culture abrogated the effects of rhTGF-β_2 on ALP activity in HBMS cells. One possible mechanism for this effect may be that rhBMP-2 decreased the number of receptors in HBMS cells, resulting in a partial loss of response to TGF-β_2. Whether BMP-2 affects TGFβ receptor expression in HBMS cells warrants further investigation.

Although rhBMP-2 and rhTGF-β_2 regulate differentially HBMS cell proliferation and differentiation, and modulate the cellular response to each other, none of the combination tested, either with rhBMP-2 and rhTGF-β_2 or with sequential treatments, led HBMS cells to form bone-like nodular structures. Other combinations of agents, or other local factors may thus be required to induce complete osteogenesis in culture of HBMS cells. Our data show however, that rhTGF-β_2 and rhBMP-2 interact with each other and act at distinct stages to induce the proliferation and differentiation of HBMS cells. Based on the present results, it is tempting to speculate that during human bone remodelling, TGF-β_2 released and activated during the bone resorption process, can stimulate the proliferation of osteoblast precursor cells in the marrow stroma and promote matrix deposition at sites of resorption. Thereafter, BMP-2 produced locally may act to reduce the effects of TGF-β_2 and to promote osteoblast differentiation. The formation of a mineralized bone matrix may therefore result from the coordinate action and interactions of TGF-β_2 and BMP-2 acting in a sequential manner on human bone marrow stromal cells at different stages of differentiation during the osteogenic process. It will be of interest to determine whether TGF-β_2 and BMP-2 act on different cell populations within the marrow stroma. Our current attempts to develop clonal human stromal cell lines at different stages of maturation [Fromigué et al., 1996; Oyajobi et al., 1997] should facilitate the identification of target cells along the osteoblast lineage in the human marrow stroma.

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REFERENCES

Amédée J, Bareille R, Rouais F, Cunningham N, Reddi H, Harmand MF (1994): Osteogenin (bone morphogenetic protein 3) inhibits proliferation and stimulates differentiation of osteoprogenitors in human bone marrow. Differentiation 58:157–164.

Beresford JN, Joyner CJ, Devlin C, Triffitt JT (1994): The effects of dexamethasone and 1,25-dihydroxyvitamin D on osteogenic differentiation of human marrow stromal cells in vitro. Arch Oral Biol 39:941–947.

Bonewald LF, Dallas SL (1994): Role of active and latent transforming growth factor beta in bone formation. J Cell Biochem 55:350–357.

Breen EC, Ignotz RA, McCabe L, Stein JL, Stein G, Lian JB (1994): TGFβ alters growth and differentiation related gene expression in proliferating osteoblasts in vitro, preventing development of the mature bone phenotype. J Cell Physiol 160:323–335.

Celeste AJ, Iannazzi JA, Taylor RC, Hewick RM, Rosen V, Wang EA, Wozney JM (1990): Identification of transforming growth factor β family members present in bone-inductive protein purified from bovine bone. Proc Natl Acad Sci USA 87:9843–9847.

Centrella M, McCarthy TL, Canalis E (1987): Transforming growth factor β is a bifunctional regulator of replication and collagen synthesis in osteoblast-enriched cell cultures from fetal rat bone. J Biol Chem 262:2869–2874.

Centrella M, Horowitz MC, Wozney JM, McCarthy TL (1994): Transforming growth factor β gene family members and bone. Endocr Rev 15:27–35.

Chen TL, Bates RL, Dudley A, Hammonds RG Jr, Amento EP (1991): Bone morphogenetic protein-2b stimulation of growth and osteogenic phenotypes in rat osteoblast-like cells: Comparison with TGF-β1. J Bone Miner Res 6:1387–1393.

Cheng SL, Yang JW, Rifas L, Zhang SF, Avioli LV (1994): Differentiation of human bone marrow osteogenic stromal cells in vitro: Induction of the osteoblast phenotype by dexamethasone. Endocrinology 134:277–286.

Dieudonné SC, Semeins CM, Goei SW, Vukicevic S, Nulend JK, Sampath TK, Helder M, Burger EH (1994): Opposite effects of osteogenin protein and transforming growth factor β on chondrogenesis in cultured long bone rudiments. J Bone Miner Res 9:771–780.

Friedenstein AJ (1976): Precursor of mechanocytes. Int Rev Cytol 47:327–355.

Fromigué O, Hott M, Marie PJ, Lomri A (1996): Establishment and characterization of a new osteoblastic cell line derived from fetal human bone marrow stroma after functional insertion of the SV-40 large T oncogen. J Bone Miner Res 11:S388 (abst).

Fromigué O, Marie PJ, Lomri A (1997): Differential effects of transforming growth factor-β, dexamethasone and 1,25-dihydroxyvitamin D on human bone marrow stromal cells. Cytokine 9:613–623.

Gehron-Robey P, Young MF, Flanders KC, Roche NS, Kondaiah P, Redi AH, Termine JD, Sporn MB, Roberts AB (1987): Osteoblasts synthesize and respond to transforming growth factor type β (TGF-β) in vitro. J Cell Biol 105:457–463.

Harris SE, Bonewald LF, Harris MA, Sabatini M, Dallas S, Feng JQ, Ghosh-Choudhury N, Wozney JM, Mundy GR (1994): Effects of transforming growth factor β on bone nodule formation and expression of bone morphogenetic protein 2, osteocalcin, osteopontin, alkaline phosphatase, and type I collagen mRNA in long-term cultures of fetal rat calvarial osteoblasts. J Bone Miner Res 9:855–863.

Hiraki Y, Inoue H, Shigeno C, Sanma Y, Bentz H, Rosen DM, Asada A, Suzuki F (1991): Bone morphogenetic proteins (BMP-2 and BMP-3) promote growth and expression of the differentiated phenotype of rabbit chondrocytes and osteoblastic MC3T3-E1 cells in vitro. J Bone Miner Res 6:1373–1385.

Hugues FJ, Collyer J, Stanfield M, Goodman SA (1995): The effects of bone morphogenetic protein-2, -4 and -6 on differentiation of rat osteoblast cells in vitro. Endocrinology 136:2671–2677.

Ignotz RA, Massague J (1986): Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extracellular matrix. J Biol Chem 261:4337–4345.

Ingram RT, Bonde SK, Riggs BL, Fitzpatrick LA (1994): Effects of transforming growth factor beta (TGF-β) and 1,25 dihydroxyvitamin D3 on the function, cytochemistry and morphology of normal human osteoblast-like cells. Differentiation 55:153–163.

Katagiri T, Yamaguchi A, Ikeda T, Yoshiki S, Wozney JM, Rosen V, Wang EA, Tanaka H, Omura S, Suda T (1990): The non-osteogenic mouse pluripotent cell line C3H10T1/2 is induced to differentiate into osteoblastic cells by recombinant human bone morphogenetic protein-2. Biochem Biophys Res Commun 172:295–299.

Kingsley DM (1994): The -β superfamily: New members, new receptors, and new genetic tests of function in different organisms. Genes and development 8:133–146.

Linkhart TA, Mohan S, Baylink DJ (1996): Growth factors for bone growth and repair: IGF, TGF-β and BMP. Bone 19:1S–12S.

Lomri A, Marie PJ (1990): Effects of transforming growth factor type β on expression of cytoskeletal proteins in endosteal mouse osteoblastic cells. Bone 11:445–451.

Long MW, Robinson JA, Ashcraft EA, Mann KG (1995): Regulation of human bone marrow-derived osteoprogenitor cells by osteogenic growth factors. J Clin Invest 95: 881–887.

Machwate M, Zerath E, Holy X, Hott M, Godet D, Lomri A, Marie PJ (1995): Systemic administration of transforming growth factor-β2 prevents the impaired bone formation and osteopenia induced by unloading in rats. J Clin Invest 96:1245–1253.

Marie PJ (1997): Effects of bone morphogenetic proteins on cells of the osteoblastic lineage. J Cell Eng 2(3):92–99.

Noda M, Camillière JJ (1989): In vivo stimulation of bone formation by transforming growth factor-β. Endocrinology 124:2991–2994.

Owen ME (1988): Marrow stromal stem cells. J Cell Sci (suppl 0):63–76.

Oyajobi BO, Lomri A, Hott M, Marie PJ (1997): A novel immortalized STRO-1+, ALP- osteoprogenitor cell line established from an immunoselected population of fetal human bone marrow cells. J Bone Miner Res 12:S201 (abst).

Pfeilschifter J, Oechsner M, Nauman A, Gronwald RGK, Minne HW, Ziegler R (1990): Stimulation of bone matrix apposition in vitro by local growth factors: A comparison between insulin-like growth factor I, platelet-derived growth factor, and transforming growth factor β. Endocrinology 127:69–75.

Rickard DJ, Sullivan TA, Shenker BJ, Leboy PS, Kazhdan I (1994): Induction of rapid osteoblast differentiation in rat bone marrow stromal cell culture by dexamethasone and BMP-2. Dev Biol 161:218–228.

Sampath TK, Maliakal JC, Hauschka PV, Jones WK, Sasak H, Tucker RF, White KH, Coughlin JE, Tucker MM, Pang RHL, Corbett C, Ozkaynak E, Oppermann H, Rueger DC (1992): Recombinant human osteogenic protein-1 (hOP-1) induces new bone formation in vivo with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation in vitro. J Biol Chem 26:20352–20362.

Thies RS, Bauduy M, Ashton BA, Kurtzberg L, Wozney JM, Rosen V (1992): Recombinant human bone morphogenetic protein-2 induces osteoblastic differentiation in W-20-17 stromal cells. Endocrinology 130:1318–1324.

Urist MR (1965) Bone formation by autoinduction. Science 150:893–899.

Wang EA, Rosen V, D'Alessandro JS, Bauduy M, Cordes P, Harada T, Israel D, Hewick RM, Kerns K.M, Lapan P, Luxenberg DP, Mcquaid D, Moutsatsos IK, Nove J, Wozney JM (1990): Recombinant human bone morphogenetic protein induces bone formation. Proc Natl Acad Sci USA 87:2220–2224.

West DC, Sattar A, Kumar S (1985): A simplified in situ solubilization procedure for the determination of DNA and cell number in tissue cultured mammalian cells. Anal Biochem 147:289–295.

Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA (1988): Novel regulators of bone formation: Molecular clones and activities. Science 242:1528–1534.

Wozney JM (1992): The bone morphogenetic protein family and osteogenesis. Mol Rep Dev 32:160–167.

Yamaguchi A, Ishizuya T, Kintou N, Wada Y, Katagiri T, Wozney JM, Rosen V, Yoshiki S (1996): Effects of BMP-2, BMP-4, and BMP-6 on osteoblastic differentiation of bone marrow-derived stromal cell lines, ST2 and MC3T3-G2/PA6. Biochem Biophys Res Commun 220:366–371.