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Expression of Bone Matrix Proteins During Dexamethasone-Induced Mineralization of Human Bone Marrow Stromal Cells

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Abstract Glucocorticoids have been shown to induce the differentiation of bone marrow stromal osteoprogenitor cells into osteoblasts and the mineralization of the matrix. Since the expression of bone matrix proteins is closely related to the differentiation status of osteoblasts and because matrix proteins may play important roles in the mineralization process, we investigated the effects of dexamethasone (Dex) on the expression of bone matrix proteins in cultured normal human bone marrow stromal cells (HBMSC). Treatment of HBMSC with Dex for 23 days resulted in a significant increase in alkaline phosphatase activity with maximum values attained on day 20 at which time the cell matrix was mineralized. Northern blot analysis revealed an increase in the steady-state mRNA level of alkaline phosphatase over 4 weeks of Dex exposure period. The observed increase in the alkaline phosphatase mRNA was effective at a Dex concentration as low as 10^{-10} M with maximum values achieved at 10^{-8} M. In contrast, Dex decreased the steady-state mRNA levels of both bone sialoprotein (BSP) and osteopontin (OPN) over a 4 week observation period when compared to the corresponding control values. The relative BSP and OPN mRNA levels among the Dex treated cultures, however, showed a steady increase after more than 1 week exposure. The expression of osteocalcin mRNA which was decreased after 1 day Dex exposure was undetectable 4 days later. Neither control nor Dex-treated HBMSC secreted osteocalcin into the conditioned media in the absence of $1,25(OH)_2D_3$ during a 25-day observation period. The accumulated data indicate that Dex has profound and varied effects on the expression of matrix proteins produced by human bone marrow stromal cells. With the induced increment in alkaline phosphatase correlating with the mineralization effects of Dex, the observed concomitant decrease in osteopontin and bone sialoprotein mRNA levels and the associated decline of osteocalcin are consistent with the hypothesis that the regulation of the expression of these highly negatively charged proteins is essential in order to maximize the Dex-induced mineralization process conditioned by normal human bone marrow stromal osteoprogenitor cells.

Key words: glucocorticoid, alkaline phosphatase, osteopontin, osteocalcin, bone sialoprotein

It has been well documented that bone marrow stromal cells include actively proliferating osteoprogenitor cells [Beresford, 1989; Owen and Friedenstein, 1988; Howlett et al., 1986; Cheng et al., 1994; Herbertson and Aubin, 1995]. We and others have previously demonstrated that human bone marrow stromal cells can be induced to differentiate into cells exhibiting osteoblast phenotype by dexamethasone (Dex) [Cheng et al., 1994; Vilamitjana-Amedee et al., 1993; Haynesworth et al., 1992]. The expression of bone matrix proteins is conditioned by the stages of osteoblastic differentiation and species [Stein et al., 1990a; Stein et al., 1990b; Owen et al., 1990; Ibaraki et al., 1992; Quarles et al., 1992; Collin et al., 1992; Yao et al., 1994]. Collagen appears earliest followed by alkaline phosphatase during the proliferation stage. At the onset of matrix mineralization, osteocalcin, osteopontin, and bone sialoprotein are expressed in large quantities and usually parallel onset of mineralization in fetal calvarial cell system. Glucocorticoids which can either stimulate, inhibit, or effect the expression of alkaline phosphatase and collagen in a biphasic fashion depending on the osteoblast systems and culture conditions [Cheng et al., 1994; Canalis, 1993; Kim and Chen, 1989; Hahn et al., 1984; Majeska et al., 1985; Ng et al., 1989; Shalhoub et al., 1992; Green et al., 1990; Subramaaniam et al., 1992;

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Kasugai et al., 1991; Leboy et al., 1991; Dietrich et al., 1979; Wong et al., 1990], can also either stimulate or inhibit the expression of osteopontin [Shalhoub et al., 1992; Yoon et al., 1987; Nagata et al., 1989]. Osteocalcin expression can be stimulated, inhibited or not affected by glucocorticoids [Shalhoub et al., 1992; Subramaaniam et al., 1992; Kasugai et al., 1991; Leboy et al., 1991] in osteoblasts whereas the expression of bone sialoprotein is consistently increased by dexamethasone in rat cells [Kasugai et al., 1991; Leboy et al., 1991; Oldberg et al., 1989]. Since Dex induced human bone marrow stromal cells to differentiate into a mineralizing osteoblast phenotype [Cheng et al., 1994; Vilamitjana-Amedee et al., 1993; Haynesworth et al., 1992], we analyzed the expression of bone matrix proteins during Dex-induced differentiation and mineralization of human bone marrow stromal cells.

MATERIALS AND METHODS

Bone Marrow Stromal Cell Culture

Human bone marrow stromal cells (hBMSC) were isolated from either male or female patients as described [Cheng et al., 1994]. Their ages ranged from 9 to 77 years old (mean ± SEM, 48.2 ± 6.1) for the male and from 32 to 68 years old (mean ± SEM, 54.3 ± 4.5) for the female patients. No differences in results were detected based on age, sex, or menopausal status of the patients. The medications used by these patients have no adverse effects on the function of osteoblastic cells. All assays were performed on first passaged cells. For short-term time course (1–7 days) and dose curve analyses, the cells were seeded and allowed to grow for 6–7 days before treatment. For long-term time course analyses (1–4 weeks), the cells were treated 3 days after seeding. In cultures treated with dexamethasone in the presence of β-glycerophosphate and ascorbic acid, minceralization was observed after 2–3 weeks exposure [Cheng et al., 1994] and was verified under phase contrast microscope.

Analysis of Alkaline Phosphatase Activity

Cells were seeded into 24-well plates at a density of 2×10^4/well and treated with dexamethasone (10^{-7} M) or ethanol vehicle. The cells were fed twice per week with media containing Dex or ethanol and harvested at indicated time. At the end of treatment, the medium was removed and the cell layers were washed and assayed for alkaline phosphatase activity by measuring *P*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) hydrolysis as previously described [Cheng et al., 1994]. Alkaline phosphatase activities were normalized by the cellular protein concentration and expressed as nmoles/min/mg protein.

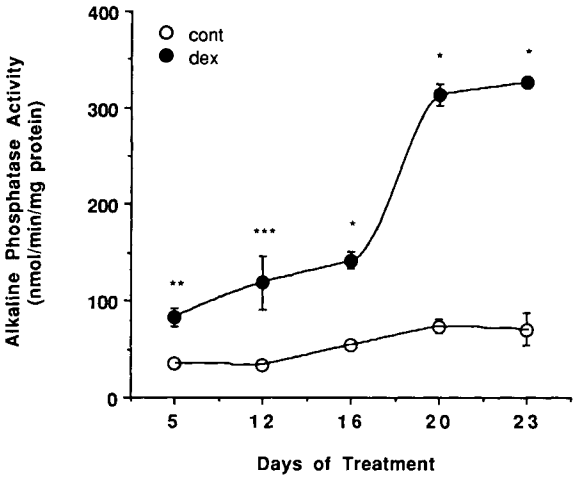

Fig. 1. Effect of dexamethasone on the alkaline phosphatase activity in human bone marrow stromal cell. HBMSC were seeded in 24 well plates. One day later (day 0) the cells were treated with either ethanol vehicle or Dex at 10^{-7} M for the indicated period of time in α-MEM containing 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate. The cells were fed twice weekly with medium containing fresh vehicle or Dex. Alkaline phosphatase activities in the cell layer were measured and normalized to the concentration of cellular protein as described in Materials and Methods. Each point (mean ± SEM) was derived from triplicate cultures. ○, control; ● Dex. $*P < 0.001$; $**P < 0.01$; $***P < 0.05$ (compared to the corresponding control value).

Osteocalcin Radioimmunoassay

Human bone marrow stromal cells in 24-well plates were treated with either vehicle or Dex (10^{-7} M) for indicated period of time. Three days before harvest, cells were washed with serum-free medium and incubated in αMEM-0.05% BSA with or without Dex as indicated. The conditioned media were harvested after 72 h incubation and stored at −80° until assay for osteocalcin (BGP) content by RIA using the kit obtained from INCSTAR Co. (Stillwater, MN). The cell layers were washed three times with PBS, scraped into 0.5 ml PBS, and sonicated. The protein concentrations in the sonicates of cell layers were measured by the Coomassie blue reagent obtained from BioRad using bovine se-

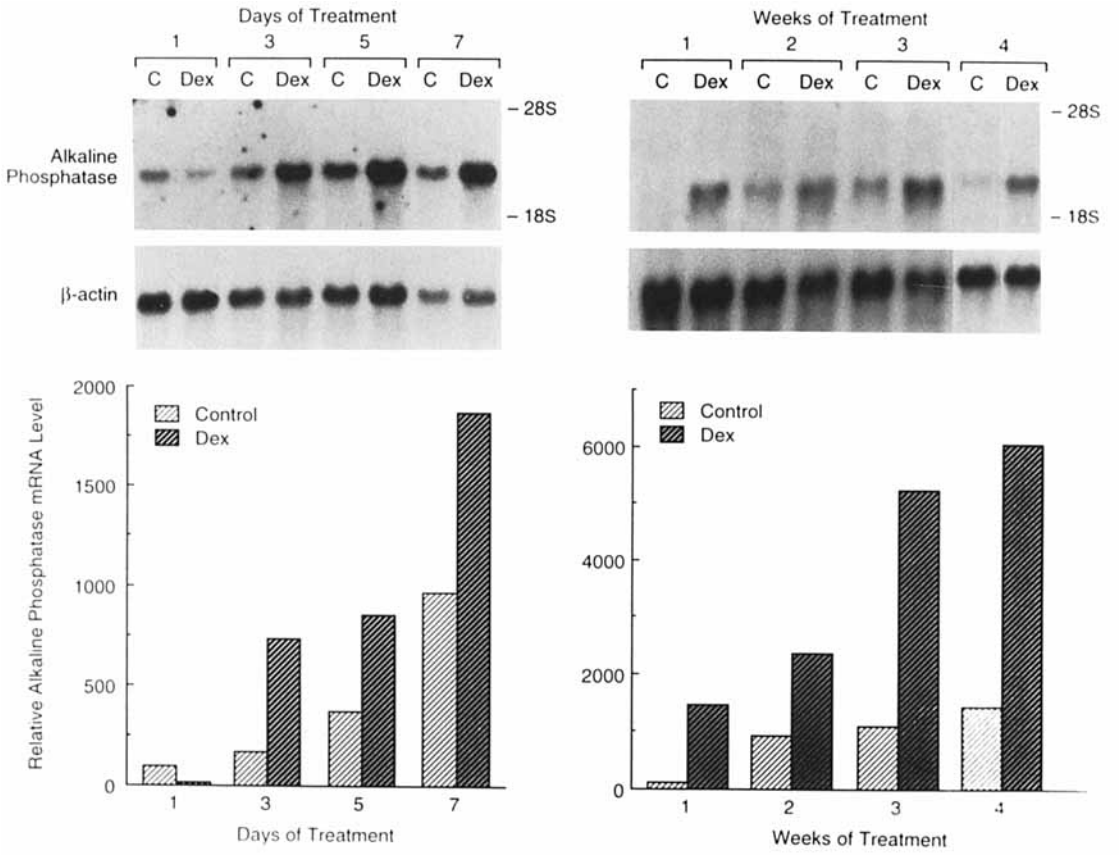

Fig. 2. Effect of dexamethasone on the steady-state mRNA levels of alkaline phosphatase in human bone marrow stromal cells. HBMSC were treated with ethanol (C) or Dex at 10^{-7} M for 1–7 days (**left**) or for 1–4 weeks (**right**). mRNA-enriched RNA preparations were isolated using Mini RiboSep kit and separated on formaldehyde agarose gel. The RNAs were transferred to a nitrocellulose or nylon membrane and probed with ^{32}P-labeled cDNA for human alkaline phosphatase followed by ^{32}P-labeled cDNA for human β-actin. Each picture shown here was representative of three experiments performed on bone marrow stromal cells derived from different patients. **Top:** Expression of these mRNAs as visualized by autoradiograms. **Bottom:** The relative alkaline phosphatase mRNA concentration on the autoradiograms as analyzed by densitometer and normalized with the β-actin mRNA level. The ratio of alkaline phosphatase/β-actin for the control culture after 1-day (**left**) or 1-week (**right**) treatment was defined as 100. *Light bar,* control cultures; *dark bar,* Dex-treated cultures.

rum albumin as standard. Osteocalcin concentration was expressed as ng/100 μg cellular protein.

Northern Blot Analysis

Human bone marrow stromal cells in *P*-150 culture dishes were treated with ethanol (control) or dexamethasone (10^{-7} M) for the indicated period of time. Poly A+ mRNA enriched RNA preparation, which still contained a substantial amount of rRNA, was isolated using Mini RiboSep mRNA isolation kit (Collaborative Biomedical Products, Bedford, MA) according to the instruction of the manufacturer. mRNAs were separated on formaldehyde-containing agarose gels, stained with ethidium bromide to obtain a picture of the 28 S and 18 S levels, and transferred to nitrocellulose or nylon membranes according to standard procedures [Ausubel et al., 1991]. The membranes were prehybridized followed by hybridization with [^{32}P]-cDNA probe overnight at 42° as previously described [Cheng et al., 1994]. [^{32}P]-cDNAs were prepared using the Megaprime Labeling kit obtained from Amersham (Arlington Heights, ILL) and 5′-[α^{32}P]-dCTP (3000 Ci/mmol, aqueous solution) according to the procedures provided by the manufacturer. Following two washes with 2× SSC/0.1% SDS for 15 min each at room temperature and a single wash with 0.2× SSC/0.1% SDS at 52°, the membranes were exposed to Hyperfilms (Amersham Co.) at −70°. To analyze the intensity of each band on the autoradiograms, the X-ray films were subjected to image analysis using ISS SepraScan 2001 (Integrated Separation Systems, Natick, MA). The relative matrix protein mRNA concentration was calcu-

lated after normalization with their respective β-actin mRNA level. Similar results were obtained when the data were normalized with eithdium bromide-stained 28 S or 18 S rRNA level or with GAPDH mRNA level. Therefore, only the data normalized with β-actin mRNA level were presented. We have not observed any changes in β-actin or GAPDH mRNA level as cultures prolonged under our experimental conditions.

Statistics

Statistical analyses were performed using Student's unpaired *t*-test.

RESULTS

Effect of Dexamethasone on Alkaline Phosphatase in Human Bone Marrow Stromal Cells

We previously reported that Dex enhanced the alkaline phosphatase activity in human bone marrow stromal cells after 2–3 days treatment and reached maximum at 7–14 days [Cheng et al., 1994]. We have since analyzed the alkaline phosphatase activities in cultures that had been exposed to Dex for over 3–4 week period. As reported earlier, alkaline phosphatase activity in HBMSC was increased by 2–3 fold after 1–2 weeks treatment. This activity was further increased to 4–5 fold of the control values by 10^{-7} M Dex between days 16 and 20 and maintained at this plateau level afterward (Fig. 1). As noted in Figure 1, alkaline phosphatase activity in the control cultures was increased when culture period was lengthened and by day 20–23, it was twice that of day 5 level. The alkaline phosphatase activity in the control cultures, however, never reached those of Dex-treated cultures.

Northern blot analysis indicated that the steady-state alkaline phosphatase mRNA levels in the control cultures progressively increased as the culture period lengthened (Fig. 2). Treatment with 10^{-7} M Dex for 1 day resulted in a drastic decline in the steady-state alkaline phosphatase mRNA level to 16–55% of the corresponding control level (Fig. 2). After 3 days treatment, however, alkaline phosphatase mRNA level rebounded to above the control value and by 7 days Dex exposure, it was 422.0 ± 135.3% (n = 10, $P < 0.001$) of the corresponding control cultures and this increase continued over 4 week period (Fig. 2). The increase in alkaline phosphatase mRNA level induced by Dex after 7 days was significant at 10^{-10} M and reached maximum value at 10^{-8} M concentration (Fig. 3).

Effect of Dexamethasone on Osteocalcin in Human Bone Marrow Stromal Cells

Although osteocalcin was not detected in the conditioned medium of either control or Dex-treated HBMSC over a 25-day period in the absence of $1,25(OH)_2D_3$ (data not shown), Northern blot analysis indicated that the osteocalcin mRNA level was decreased by Dex after 1 day exposure to 11–45% of the control value with a further decrease observed during the subsequent days reaching undetectable levels after 4 days of treatment (Fig. 4). This inhibition by

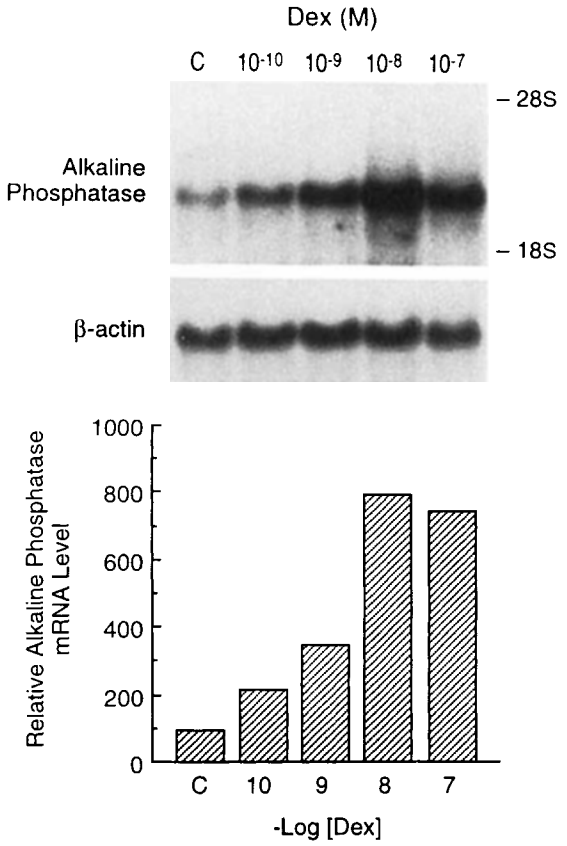

Fig. 3. Effect of dexamethasone on the steady-state mRNA levels of alkaline phosphatase in human bone marrow stromal cells as a function of dose. HBMSC were treated with ethanol (C) or Dex at indicated concentration for 7 days. Northern blot analysis was performed as described. The picture shown here was representative of three experiments performed on bone marrow stromal cells derived from different patients. **Top:** Expression of these mRNAs as visualized by autoradiograms. **Bottom:** The relative alkaline phosphatase mRNA concentration on the autoradiograms as analyzed by densitometer and normalized with the β-actin mRNA level. The ratio of alkaline phosphatase/β-actin for the control culture was defined as 100.

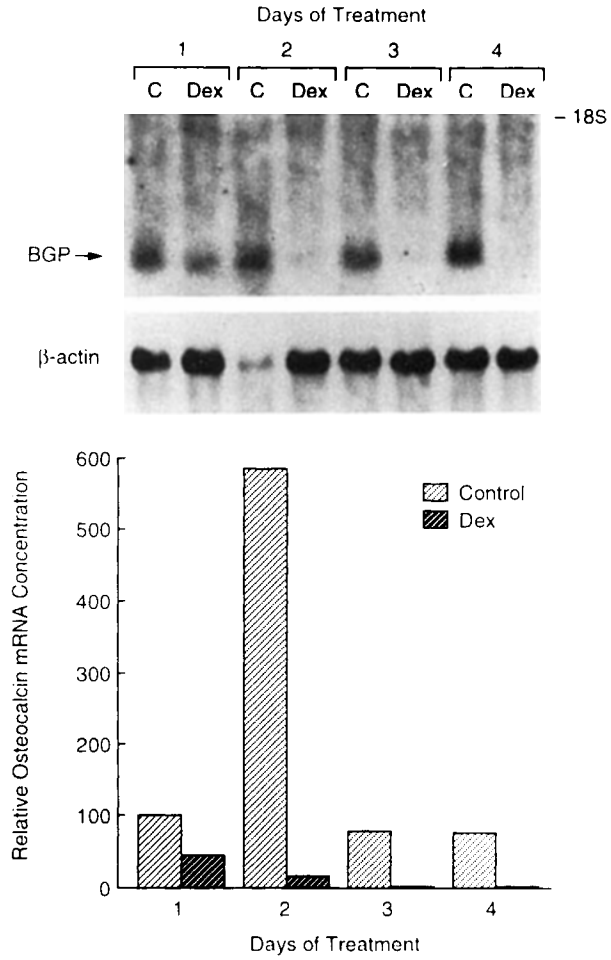

Fig. 4. Effect of dexamethasone on the steady-state mRNA levels of osteocalcin (BGP) in human bone marrow stromal cells. HBMSC were treated with ethanol (C) or Dex at 10^{-7} M for 1–4 days. mRNA-enriched RNA preparations were isolated and membrane was probed with ^{32}P-labeled cDNA for human osteocalcin followed by ^{32}P-labeled cDNA for human β-actin. The picture shown here was representative of two experiments performed on bone marrow stromal cells derived from different patients. **Top:** Expression of these mRNAs as visualized by autoradiograms. **Bottom:** The relative osteocalcin mRNA concentration on the autoradiograms as analyzed by densitometer and normalized with the β-actin mRNA level. The ratio of osteocalcin/β-actin for the control culture after 1-day treatment was defined as 100. *Light bar,* control cultures; *dark bar,* Dex-treated cultures.

Dex persisted over 4 weeks period and was effective at 1 nM concentration (data not shown).

Since the protein concentration of osteocalcin and enzyme activity of alkaline phosphatase in general paralleled their respective mRNA level, we employed only Northern blot analysis to analyze the regulation of osteopontin and bone sialoprotein in HBMSC.

Effect of Dexamethasone on the Expression of Osteopontin in Human Bone Marrow Stromal Cells

Osteopontin mRNA level in the control culture was increased more than two fold after 7 days and continued to increase to almost five fold of the 1-week level after 4 weeks (Fig. 5). Dex decreased the steady-state osteopontin mRNA level to 49.6 ± 16.6% (n = 5, $P < 0.01$) 1 day after exposure and to 11.2 ± 6.3% (n = 5, $P < 0.001$) of the control level by 7 days (Fig. 5). The reduction of osteopontin mRNA level by Dex persisted over four weeks observation period (Fig. 5). Although Dex-treated cultures expressed considerably lower osteopontin mRNA concentration than the control cultures, the relative osteopontin mRNA levels among the Dex-treated group revealed an increase of 17 fold from week 1 to week 4 (Fig. 5). Dose response analyses indicated that the inhibition of osteo-

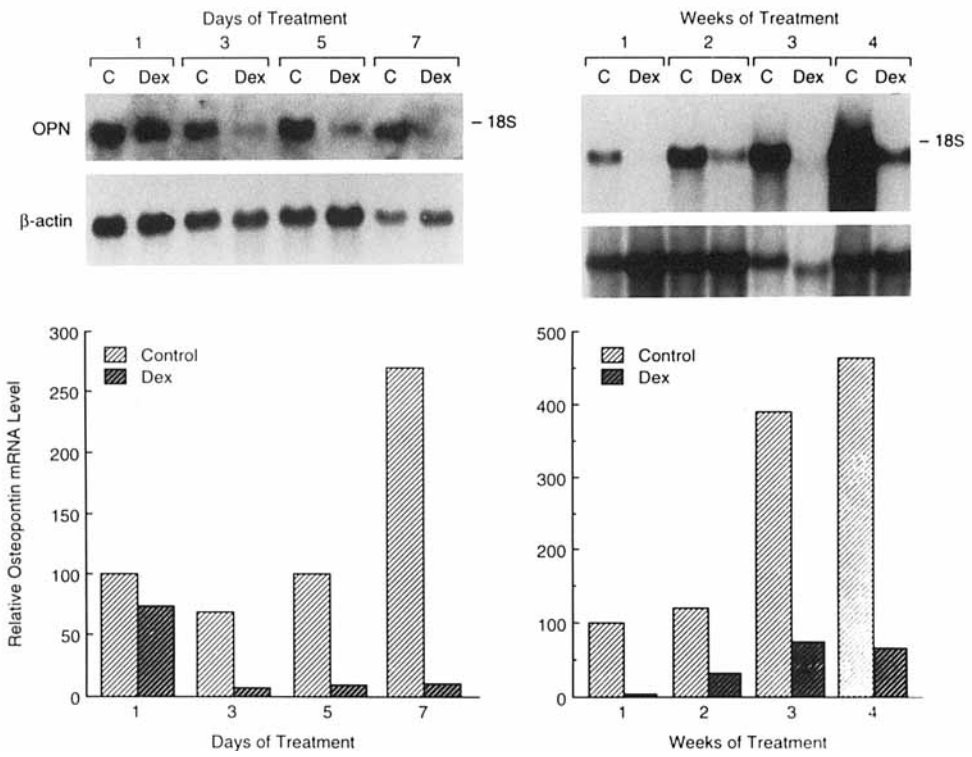

Fig. 5. Effect of dexamethasone on the steady-state mRNA levels of osteopontin in human bone marrow stromal cells. HBMSC were treated with ethanol (C) or Dex at 10^{-7} M for 1–7 days **(left)** or 1–4 weeks **(right)**. Northern blot analysis was performed and membranes were probed with ^{32}P-labeled cDNA for human osteopontin followed by ^{32}P-labeled cDNA for human β-actin. The pictures shown here were representative of three (left) and two (right) experiments performed on bone marrow stromal cells derived from different patients. **Top:** Expression of these mRNAs as visualized by autoradiograms. **Bottom:** The relative osteopontin mRNA concentration on the autoradiograms as analyzed by densitometer and normalized with the β-actin mRNA level. The ratio of osteopontin/β-actin for the control culture after 1-day (left) or 1-week (right) treatment was defined as 100. *Light bar,* control cultures; *dark bar,* Dex-treated cultures.

pontin mRNA level by Dex was effective at 10^{-10} M and reached maximum at 10^{-8} M (Fig. 6).

Effect of Dexamethasone on the Expression of Bone Sialoprotein in Human Bone Marrow Stromal Cells

Similar to osteopontin, bone sialoprotein mRNA level in the control culture was increased steadily over 3 weeks period (Fig. 7). Dex treatment resulted in a decreased expression of bone sialoprotein mRNA to 20.5 ± 6.3% (n = 8, $P < 0.001$) of the corresponding control value by 7 days and persisted over a 3 week period (Fig. 7). Although Dex-treated cultures expressed lower bone sialoprotein mRNA than the control cultures, the relative bone sialoprotein mRNA levels among the Dex cultures showed an increase of 10-fold over a 3 week period (Fig. 7), a phenomenon also observed for osteopontin (Fig. 5). In another experiment, the level of BSP mRNA was found to continue to increase 4 weeks after Dex treatment although it was still substantially lower than the control value (data not shown). Dose response analysis indicated that the inhibition of bone sialoprotein mRNA level by Dex was effective at 10^{-9} M and reached maximum at 10^{-8} M (Fig. 8).

Summary of the Effect of Dex on the Matrix Protein mRNA Expression During the Differentiation of Human Bone Marrow Stromal Cells

Figure 9 summarized the effect of Dex on the expression of alkaline phosphatase, osteopontin, bone sialoprotein, and osteocalcin over the time course of differentiation and mineralization of human bone marrow stromal cells and their comparison with that of control cultures. As shown in the figure, maximum expression of alkaline phosphatase, osteopontin, and bone sialoprotein in either control or Dex-treated group was observed after 3 or 4 weeks. Exposure to

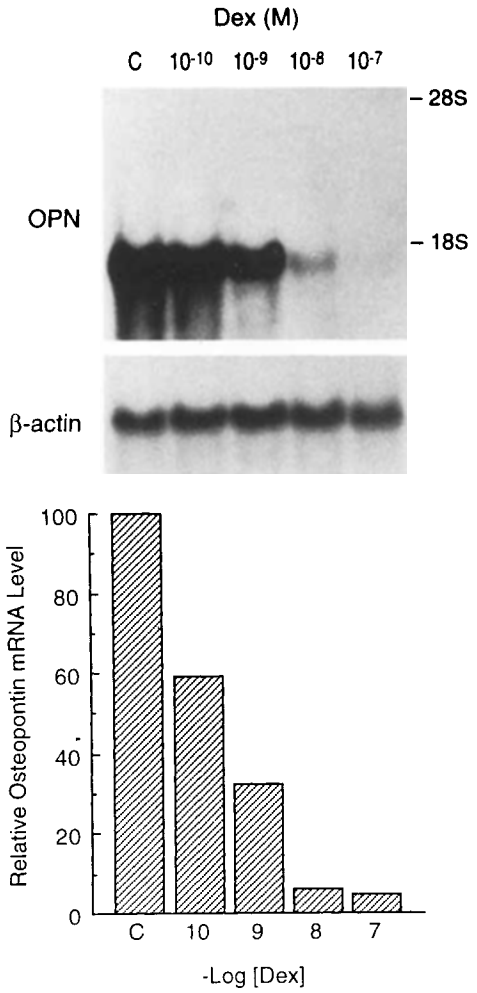

Fig. 6. Effect of dexamethasone on the steady-state mRNA levels of osteopontin in human bone marrow stromal cells as a function of dose. HBMSC were treated with ethanol (C) or Dex at indicated concentration for 7 days. Cells were harvested and Northern blot analysis performed. Membrane was probed with ^{32}P-labeled cDNA for human osteopontin (OPN) followed by ^{32}P-labeled cDNA for human β-actin. The result shown here was representative of two experiments performed on bone marrow stromal cells derived from different patients. **Top:** Expression of these mRNAs as visualized by autoradiograms. **Bottom:** The relative osteopontin mRNA concentration on the autoradiograms as analyzed by densitometer and normalized with the β-actin mRNA level. The ratio of osteopontin/β-actin for the control culture was defined as 100.

Dex, however, resulted in a substantially higher expression of alkaline phosphatase but a drastically suppressed levels of osteopontin and bone sialoprotein in conjunction with heavy mineralization of the matrix. The expression of osteocalcin mRNA was maximum at the beginning of Dex treatment and declined to almost undetectable level after 4 days.

DISCUSSION

We and others have previously demonstrated that dexamethasone stimulates the differentiation of human bone marrow stromal cells into osteoblast-like cells with mineralization of the matrix [Cheng et al., 1994; Gronthos et al., 1994]. Since bone matrix proteins play pivotal roles in the differentiation and mineralization processes [Boskey, 1992; Gorski, 1992], we considered an analysis of the expression of the bone matrix proteins during the Dex-induced differentiation process in human bone marrow stromal cells an essential undertaking. Our results indicated that whereas dexamethasone increased alkaline phosphatase, the steady-state mRNA levels of osteopontin and bone sialoprotein were decreased and osteocalcin expression suppressed to undetectable level over a 4 week treatment period.

The enzyme activity and the steady-state mRNA level of alkaline phosphatase in Dex-treated HBMSC displayed a gradual and moderate increase in the first 2 weeks of exposure followed by a drastic increase in the third week with maximal values attained in the 4th week. It has been demonstrated that the increase in alkaline phosphatase mRNA by Dex can be attributed entirely to an increase in the transcriptional process [Green et al., 1990]. Interestingly, the control cultures of HBMSC also showed a time-dependent increase in alkaline phosphatase activity and mRNA level although neither value reached the levels attained by Dex after 1 week treatment (Figs. 1 and 2). Since only the Dex-treated cultures mineralized the matrix [Cheng et al., 1994], these observations imply that a threshold of alkaline phosphatase activity must be exceeded before mineralization can occur.

The pattern of the increase in alkaline phosphatase by Dex in HBMSC was similar to that found by others in the differentiation of MC3T3-E1 cells [Quarles et al., 1992] where the alkaline phosphatase mRNA increased continuously during the length of culture period. Our results, however, are in contrast to those found in osteoblasts derived from fetal rat calvaria and fetal bovine long bones which exhibit maximal alkaline phosphatase expression during proliferation and the matrix maturation periods with subsequent decrease during the mineralization process [Stein et al., 1990b; Owen et al., 1990; Ibaraki et al., 1992]. Unlike HBMSC, the maxi-

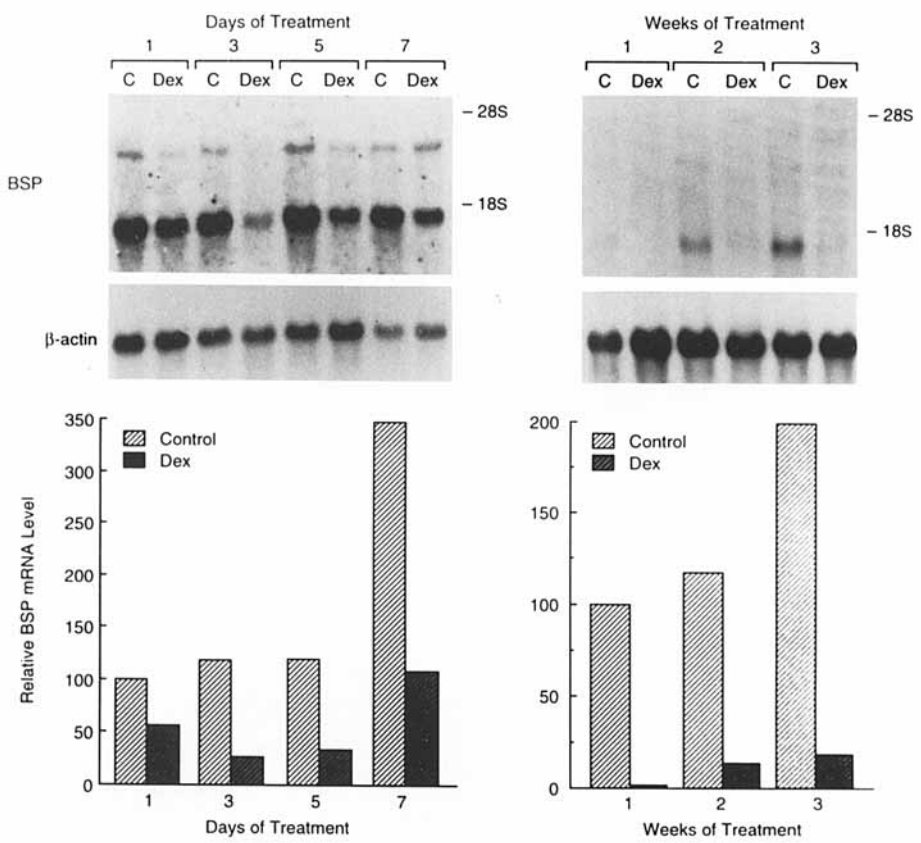

Fig. 7. Effect of dexamethasone on the steady-state mRNA levels of bone sialoprotein in human bone marrow stromal cells. HBMSC were treated with ethanol (C) or Dex at 10^{-7} M for 1–7 days (**left**) or 1–3 weeks (**right**). mRNA-enriched RNA preparations were isolated and Northern blot analysis performed. Membranes were probed with ^{32}P-labeled cDNA for human bone sialoprotein (BSP) followed by ^{32}P-labeled cDNA for human β-actin. Each picture shown here was representative of three experiments performed on bone marrow stromal cells derived from different patients. **Top:** Expression of these mRNAs as visualized by autoradiograms. **Bottom:** The relative bone sialoprotein mRNA concentration on the autoradiograms as analyzed by densitometer and normalized with the β-actin mRNA level. The ratio of bone sialoprotein/β-actin for the control culture after 1-day (**left**) or 1-week (**right**) treatment was defined as 100. *Light bar,* control cultures; *dark bar,* Dex-treated cultures.

mum expression of alkaline phosphatase in both control and Dex-treated rat bone marrow stromal cells also occurred after 2 weeks and declined thereafter [Yao et al., 1994; Malaval et al., 1994]. Thus, the behavior of human bone marrow stromal cells differ from that of rat bone marrow stromal cells in this regard.

The suppression of osteopontin, bone sialoprotein, and osteocalcin by Dex in HBMSC is inconsistent with the generally held view in that maximum expression of these three proteins is essential for mineralization to occur. Abundant evidence has accumulated indicating that osteopontin, bone sialoprotein, and osteocalcin express highly during the mineralization phase of fetal and neonatal osteoblasts and mineralized bone tissues [Stein et al., 1990b; Owen et al., 1990; Ibaraki et al., 1992; Yoon et al., 1987; Nagata et al., 1989; Bianco et al., 1993; McKee et al., 1990; Chen et al., 1991; Chen et al., 1992; Chen et al., 1993; Chen et al., 1994; Kasugai et al., 1992; Nomura et al., 1988; Nagata et al., 1991; Hultenby et al., 1991; Weinreb et al., 1990]. Bone sialoprotein is also able to induce the nucleation of hydroxyapatite in vitro [Hunter and Goldberg, 1993]. Our accumulated data, however, demonstrate that osteopontin and bone sialoprotein expression in the control cultures of HBMSC far surpasses that found in Dex-treated cultures despite the absence of mineralization [Cheng et al., 1994; Gronthos et al., 1994]. Although we have not yet fully analyzed the protein concentrations of osteopontin and bone sialoprotein in our culture systems, preliminary results suggest that the mRNA level of osteopontin correlates with its protein concentration (data

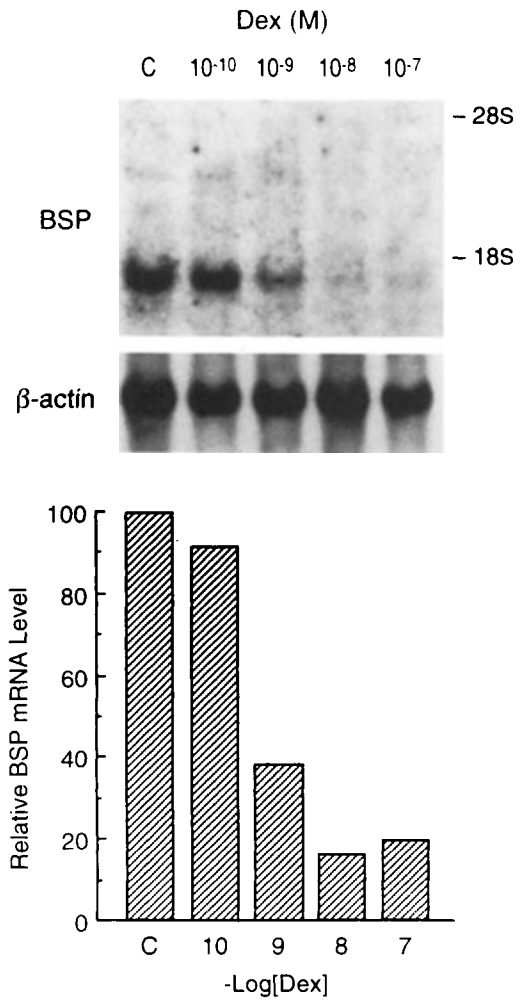

Fig. 8. Effect of dexamethasone on the steady-state mRNA levels of bone sialoprotein in human bone marrow stromal cells as a function of dose. HBMSC were treated with ethanol (C) or Dex at indicated concentration for 7 days. Northern blot analysis was performed and membrane was probed with ^{32}P-labeled cDNA for human bone sialoprotein followed by ^{32}P-labeled cDNA for human β-actin. The result shown here was representative of two experiments performed on bone marrow stromal cells derived from different patients. **Top:** Expression of these mRNAs as visualized by autoradiograms. **Bottom:** The relative bone sialoprotein mRNA concentration on the autoradiograms as analyzed by densitometer and normalized with the β-actin mRNA level. The ratio of bone sialoprotein/β-actin for the control culture was defined as 100.

not shown). Thus, in human bone marrow stromal cells subjected to dexamethasone, osteopontin, bone sialoprotein, and osteocalcin do not appear to assume the determining pivotal roles for the mineralization process. This observation is also consistent with the finding that high concentrations of osteopontin and bone sialoprotein prevent hydroxyapatite formation in in vitro assays [Boskey, 1992; Gorski, 1992]. Since Dex induces the mineralization of the matrix of human bone marrow stromal cells, the expression of appropriate levels of all the bone matrix proteins is pivotal. Our results are also consistent with the observation that the concentration of osteopontin and bone sialoprotein is relatively low in the bone formation areas in the human bone tissues [Ingram et al., 1993]. A moderate and sufficient quantity of osteopontin and bone sialoprotein is probably important in initiating and maximizing the matrix mineralization process. In addition, the degree of phosphorylation, sulfation and sialization of osteopontin, and bone sialoprotein may also affect the mineralization process [Boskey, 1992; Gorski, 1992; Nagata et al., 1991]. The possibility that Dex affects the post-translational modification of osteopontin and bone sialoprotein still remains to be explored.

Dexamethasone induced bone sialoprotein mRNA expression in fetal and neonatal rat calvarial cells, ROS 17/2.8 cells and rat bone marrow stromal cells [Kasugai et al., 1991; Leboy et al., 1991; Oldberg et al., 1989; Malaval et al., 1994]. Our accumulated data indicated that Dex decreased the steady-state mRNA levels of bone sialoprotein in human bone marrow stromal cells despite the fact that these Dex-treated cells were able to mineralize their matrix [Cheng et al., 1994; Vilamitjana-Amedee et al., 1993; Gronthos et al., 1994]. Thus, species difference or culture conditions may dictate the outcome in the expression of bone sialoprotein. A putative glucocorticoid response unit is present at −1038 to −1022 in the human BSP promoter [Kim et al., 1994]. Since this region is not conserved between human and rat, the opposite effect of Dex on the expression of BSP between human and rat BMSC indicates that different nuclear factor (or factors) may condition the Dex-mediated regulation of the transcription of BSP. Additional studies designed to analyze the identification and function of nuclear factor(s) on the promoter activities should shed some new light on the discrepant effect of Dex on human and rodent bone cells.

Osteocalcin has been shown to correlate very well with the mineralization of fetal rat calvarial cells [Stein et al., 1990b; Pockwinse et al., 1992] and Dex increases osteocalcin expression in several osteoblast systems and rat bone marrow stromal cells [Shalhoub et al., 1992; Kasugai et al., 1991; Leboy et al., 1991; Yao et al., 1994; Malaval et al., 1994]. In many other osteoblast

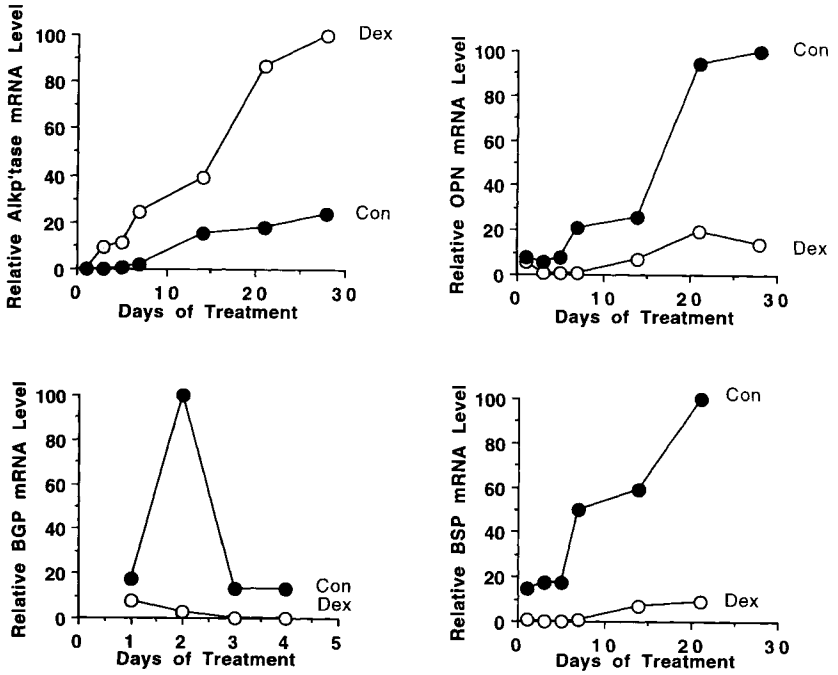

Fig. 9. Summary of the expression of alkaline phosphatase, osteopontin, bone sialoprotein, and osteocalcin mRNA levels as a function of time. The β-actin normalized mRNA level of each matrix protein was plotted as a function of treatment length. The highest value of each matrix protein for both control (Con) and Dex combined was defined as 100 and the rest of the number adjusted accordingly.

systems, however, Dex has been shown to inhibit or has no effect on the expression of osteocalcin and prevents the induction of osteocalcin by vitamin D [Kim and Chen, 1989; Subramaaniam et al., 1992; Stromstedt et al., 1991]. Using human bone marrow stromal cells, we were unable to detect osteocalcin in the conditioned medium in the absence of $1,25(OH)_2D_3$ and Dex decreased the osteocalcin mRNA level to almost undetectable levels. Since Dex induces the mineralization of the matrix of human bone marrow stromal cells, our results argue against the role of osteocalcin in the initiation of mineralization. Furthermore, inhibition of γ-carboxylation of osteocalcin has been shown to result in excessive mineralization [Price et al., 1982] and osteocalcin inhibits the growth of hydroxyapatite crystals [Romberg et al., 1986]. Therefore, osteocalcin may function as a regulator rather then a promoter for mineralization. In fact, it has been suggested that osteocalcin may play an important role in bone resorption [Glowacki et al., 1989; Glowacki and Lian, 1987]. Although we could not detect osteocalcin in the conditioned medium, it is possible that our techniques were not sensitive enough to detect levels which albeit very low, are essential for the propagation of mineralization. Further studies using more sensitive immunohistochemical staining will clarify this aspect.

It has been reported that Dex induces differentiation of bone marrow stromal cells to adipocytes in the presence of methyisobutylxanthine and indomethacin [Gimble, 1990]. These cells also express alkaline phosphatase, osteopontin, bone sialoprotein, and osteocalcin [Dorheim et al., 1993]. In contrast to our findings, however, the Dex-induced adipocytes exhibit lower alkaline phosphatase with no alteration in osteopontin mRNA levels. Therefore, Dex per se appears unable to direct the differentiation of human bone marrow stromal cells toward adipocytes in our culture conditions, confirming our earlier observation that Dex-treated human bone marrow stromal cells contain no adipocytes [Cheng et al., 1994].

In conclusion, although individuals subjected to prolonged high-dose glucocorticoid therapy can lose bone mass [Lukert and Raisz, 1990], our in vitro data together with observations of others made in human osteoblasts [Subramaaniam et al., 1992; Wong et al., 1990] strongly suggest that glucocorticoids at physiological concentration play an essential role in enhancing

the differentiation and mineralization of both human bone marrow stromal osteoprogenitor cells and human osteoblasts by maintaining the expression of specific matrix protein components at appropriate levels.

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