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Phenotypic Effects of Continuous or Discontinuous Treatment With Dexamethasone and/or Calcitriol on Osteoblasts Differentiated From Rat Bone Marrow Stromal Cells

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Abstract Osteoblasts are target cells for glucocorticoids and calcitriol, and their phenotype is greatly modified by these hormones. We investigated the effect of continuous or discontinuous hormonal exposure to osteoblasts derived from rat bone marrow stromal cells in long-term subcultures. Stromal cells were grown in primoculture in presence of dexamethasone (dex), but in following subcultures, dex and/or calcitriol were added just after seeding or after a 7-day hormone-free period. Cell proliferation, alkaline phosphatase (ALP) histochemical staining, and enzymatic bioactivity measurement, osteocalcin (OC), ALP and bone sialoprotein (BSP) mRNA expression were used to study the differential effect on osteoblastic phenotype of various conditions of treatment by dex and calcitriol. In primoculture, the osteoblastic differentiation was confirmed by the formation of calcified nodules and by strong expression of ALP, OC, and BSP mRNAs. In subcultures, proliferation of stromal cells was stimulated by dex and inhibited by calcitriol and by both hormones. Cell proliferation was not modified by hormonal lack during 7 days. Continuous hormonal treatment by dex strongly enhanced OC and BSP mRNAs, but apparently did not modified ALP mRNAs expression. Continuous treatment by calcitriol decreased ALP and the dex-induced BSP expression and stimulated the OC mRNAs level, strongly when associated with dex. The population of ALP^+ cells and ALP bioactivity were strongly increased by dex, whereas calcitriol or both hormones decreased them. When the subcultures were undergone without hormonal treatment during 7 days, all osteogenic mRNAs strongly decreased even after hormonal recovery. Dex, calcitriol, and both hormones inhibited ALP mRNAs. OC messengers were only weakly detectable with both hormones. ALP^+ cell population and ALP bioactivity were decreased after 14 days of hormonal treatment recovery. These results support that continuous presence of glucocorticoids appears as a major key for the permanent expression of the osteoblastic phenotype that is inhibited by calcitriol, in the rat bone marrow. J. Cell. Biochem. 85: 640–650, 2002.

Key words: osteoblast; dexamethasone; calcitriol; bone marrow stromal cells; osteoblastic differentiation

Bone formation requires differentiated and active osteoblasts to synthesize the organic matrix that supports the mineralizing process. Regulation of osteoblast differentiation appears as an essential key for the maintenance of continuous supply of mature osteoblasts needed for bone growth, remodeling, or fracture repair. Osteoblast precursors are believed derived from a multipotential stem cell of the bone marrow stroma [Prockop, 1997]. However, early osteoblast precursors are included in colony termed as fibroblast colony forming units (CFU-f) and that are represented in very small amounts in total bone marrow, about $1/10^5$ marrow cells [Rickard et al., 1996].

The osteoblast phenotype is usually characterized by various markers. Alkaline phosphatase (ALP) is required for proper bone matrix mineralization. Osteocalcin (OC) was demonstrated to be a specific regulator of the length of bone mineral crystal and important for induction of the osteoclast phenotype. Bone sialoprotein (BSP) is involved in bone mineralization,

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hydroxyapatite nucleation, and osteoblast attachment via RGD sequences and $\alpha_V\beta_3$ integrin (vitronectin cell receptor). Type I collagen is the major bone matrix protein [Robey and Boskey, 1996; Shi et al., 1996; Komori et al., 1997].

The osteoblastic markers can be successively detected during the progressive sequence of osteoblast differentiation. Although, all the genes expressed in fibroblasts are also expressed in osteoblasts, and conversely, only two osteoblast-specific transcripts have been identified: one encoding OC and the other, *Cbfa* 1, a specific transcription factor, regarded as the earliest and most specific marker of osteoblast differentiation [Rodan and Harada, 1997; Ducy et al., 2000]. Type I collagen expression appears early in osteoprogenitor cells and is still going on in mature osteoblasts. ALP can be detected in precursors cells and mature osteoblasts, whereas BSP and OC are markers for mature osteoblasts [Beresford, 1997].

A lot of systemic and local hormones, growth factors, and cytokines have been shown to be involved in osteoblastic differentiation processes and activities [Fromingué et al., 1997]. Previous data suggest that osteoblast precursors in the bone marrow stroma are target cells for 1,25-$(OH)_2D_3$, which regulates cell proliferation, ALP activity, osteopontin (OP), and OC synthesis [Ishida et al., 1993; Denis et al., 2000]. Glucocorticoids were shown to induce in vitro the differentiation of rat marrow stromal cells into osteogenic cells, and in vitro, dexamethasone (dex), a synthetic glucocorticoid, increases the expression of osteoblastic markers (ALP, OP, and OC) in animal and human bone marrow stromal cells [Lian and Stein, 1993; Ohgushi et al., 1996; Peter et al., 1998]. Therefore, dex appears to be required in culture for induction of the osteoblastic phenotype in bone marrow stromal cells [Collin et al., 1992; Herbertson and Aubin, 1995].

In this study, we used osteoblasts cells derived from rat bone marrow to study the effects of dex and calcitriol on cell proliferation, differentiation, activity, and expression in long-term cultures. The aim of the study was to determine the role of these hormones in the maintenance of the osteoblastic phenotype. Cells were first cultivated in primoculture in presence of dex and subcultivated with or without a 7 day period free of hormone treatment before recovery of hormonal supplementation.

MATERIALS AND METHODS

Reagent

Dulbecco's modified Eagle medium (DMEM), antibiotics (penicillin, streptomycin, amphotericin B, gentamycin), trypsin/ethylenediamine tetraacetic acid (EDTA), fetal calf serum (FCS), and cell culture disposables (6- and 24-wells plates, T75 flasks, and 10-mm diameter dishes) were purchased from Life Technologies-GIBCO (Cergy Pontoise, France). Type IV collagenase, L-ascorbic acid, β-glycerophosphate, dex, and 1,25 $(OH)_2$ vitamin D_3 (calcitriol) were obtained from Sigma (Saint Quentin Fallavier, France), and [methyl-^{3}H] thymidine was from Pharmacia (Orsay, France). Monoclonal antibody anti-type I collagen and 20-nm colloidal gold-labeled protein A were purchased from Sigma; Alexa Fluor™ 488 goat anti-mouse IgG conjugate was from Interchim (Montluçon, France). Glutaraldehyde was obtained from Merck (Fontenay sous Bois, France) and hexamethyldizilasane from Acros Organics (Noisy le Grand, France). For ALP histochemical detection and bioactivity measurement, naphthol AS-BI, fast blue BB salt, paranitrophenyl phosphate (PNPP), and ALP buffer were purchased from Sigma, and the protein assay kit from BioRad (München, Germany). For RNA preparation and Northern blots, guanidine thiocyanate, formamide, formaldehyde, and salmon testes DNA were obtained from Sigma, nylon membranes from Appligene (Illkirch, France), agarose from Eurogentec (Herstal, Belgium). The DNA labeling kit was from Pharmacia and [^{32}P]-dCTP from NEN (Boston, MA). Rat OC (rOC) and mouse BSP (mBSP) cDNA probes were kindly provided by J. Aubin (Toronto, Ontario, Canada), and the rat ALP (rALP) cDNA probe by JL Reid, Merck & Co. (West Point, PA).

Cell Culture

Isolation of rat marrow stromal cells. Rat bone marrow were prepared and cultured as previously described [Maniatopoulos et al., 1988]. Briefly, young adult male Wistar rats (3–4 weeks) were killed by cervical dislocation. Femurs and tibias were removed under sterile conditions and immersed in DMEM with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, 300 ng/ml fungizone). After removal of the bone heads, the marrow was collected by

flushing repeatedly through the shafts with a syringe containing DMEM supplemented with antibiotics as above and 15% heat-inactivated FCS. Aggregates were removed from the cell suspension by sieving through a 70-μm cell strainer (Falcon, Grenoble, France). Cell number was determined using trypan blue staining with a Malassez cell.

Primoculture. Cells were plated into 75-cm^2 tissue flasks or six-wells plates at 10^6 cells/cm^2 density in basic medium (DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM L-ascorbic acid, and 15% FCS). Dex (10^{-8} M) was added to favor the development of the osteoblastic population. Two days after plating, non-adherent cells were removed by two vigorous washing with phosphate buffer saline (PBS), pH 7.4. Thereafter, the medium was changed twice weekly until cell harvesting. After 7 days, primocultures were extensively rinsed with serum-free DMEM, and then incubated at 37°C for 2 h in serum-free DMEM containing 25 U/ml type IV collagenase and 2 mM $CaCl_2$. Cultures were then softly washed with PBS and treated by 0.1 % trypsin-EDTA in PBS. After rinsing in PBS, cells were counted and plated in subculture conditions. For calcified nodules detection (Von Kossa staining, ALP histochemistry, and scanning electron microscopy (SEM)), primocultures were also going on for 16 days, in the same conditions as above, but supplemented with 10 mM β-glycerophosphate.

Subcultures. Cells, seeded at 5×10^3 cells/cm^2, were cultivated for 4, 7, and 14 days in basic medium with different conditions: (i) 10^{-8} M dex, (ii) 10^{-8} M 1,25-$(OH)_2$ vitamin D_3, (iii) 10^{-8} M dex and 10^{-8} M 1,25-$(OH)_2$ vitamin D_3, and (iv) hormone-free medium, but with vehicle (< 0.1% ethanol) for control condition. Moreover, subcultures were also performed for 11, 14, and 21 days in the same conditions, but with a initial 7 days period (day 0–7), during which no hormone was added to the medium culture (Fig. 1).

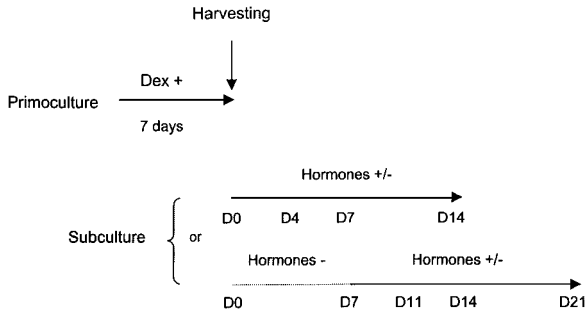

Fig. 1. Rat bone marrow stromal cells were grown for 7 days in primoculture with 10^{-8} M dex. Subcultures were treated for 14 days with 10^{-8} M dex or 10^{-8} M calcitriol or both hormones continuously with primoculture (day 4, 7, 14) or after a 7 day period free of hormonal treatment (day 11, 14, 21).

Cell Proliferation Assay

All experiments were done in triplicate for each culture condition and time, at the initial density of 5×10^3 cells/cm^2 in 0.5-ml medium. Proliferation was measured using [^{3}H] thymidine incorporation. Six hours before harvesting, 1 μCi [methyl-^{3}H] thymidine was added to each well. Incorporated isotope was measured after trichloroacetic acid precipitation in a liquid scintillation analyzer (Tri-carb 2300 TK, Packard, Meridien, CT). Results were expressed as the percentage of increase or decrease vs. control at the same time of culture.

Histochemical Staining

ALP activity was histochemically detected in six-well culture plates. Cell cultures were rinsed three times with PBS and fixed with 3.7% formaldehyde in PBS for 10 min at 37°C. After extensive washing, cells were incubated for 30 min at 37°C with 0.5 mg/ml naphthol AS-BI phosphate in Tris-buffer (pH 8.5) in the presence of 1 mg/ml fast blue BB salt. The percentage of ALP-positive (ALP^+) cells was determined under light microscope (magnification 400 ×). ALP^+ and ALP^- cells were counted in triplicate (3 culture plates, 20 running fields per culture plate, 0.16 mm^2 per field).

The presence of calcified nodules was demonstrated with the Von Kossa method. After rinsing, cells were incubated for 10 min at room temperature with 5% lithium carbonate, 15 min with 2% silver nitrate, 5 min with 0.5% hydroquinone, and 5 min with 5% sodium thiosulfate.

ALP Bioactivity

ALP bioactivity was assayed in cell lysates by determining the release of *p*-nitrophenol from PNPP substrate solution. Briefly, cell layers were washed twice in PBS and stored at − 20°C until assayed. For assay, cells were thawed and scraped into 0.5 ml of Tris-HCl, 0.1 M, pH 7.5, 0.1% Triton, and sonicated three times during 10 s. After centrifugation for 30 min at 30,000*g*, aliquots of supernatants were incubated during

20 min with 10 mM PNPP in 0.1 M 2-amino-1-methyl-1-propanol buffer, pH 10.3, supplemented with 2-mM $MgCl_2$ and the reaction was stopped with NaOH 0.5 N. Measurements were done in triplicate at 410 nm using a spectrometer (Shimatzu, Kyoto, Japan). Total protein was measured at 595 nm in similar aliquots using a protein assay kit with Coomassie blue. ALP activity was expressed as nmol/min/mg protein.

SEM Study

Cells were fixed with 2.5% glutaraldehyde in 0.1-M cacodylate buffer (pH 7.4) for 30 min at room temperature, post-fixed in 1% osmium tetraoxide in distilled water, for 30 min, dehydrated through a graded ethanol series, desiccated overnight in hexamethyldizilasane, and then carbon-coated by sputtering (Bal-Tec). Observations were done with a Jeol JSM 6301F scanning electron microscope and the Ca/P ratio in mineralized nodules (10 measurements, mean $\pm$ SD) was determined with X-ray electron microanalysis (EDX, Links, Oxford, UK).

Collagen I Immunostaining

For SEM immunogold detection of type I collagen, cells were cultivated in primoculture for 16 days on glass coverslip, fixed with freshly-prepared 2% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 30 min at room temperature and extensively washed in 0.2 M cacodylate buffer. Saturation of non-specific binding sites was performed with 50 mM NH_4Cl for 10 min and 1% bovine serum albumin (BSA) for 30 min. Cells were then incubated at room temperature in a humidified chamber with a 1/100 diluted anti-type I collagen monoclonal antibody for 2 h and then with protein A labeled with 20 nm gold particles. Cells were then post-fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 30 min and in 1% osmium tetraoxide for additional 30 min, dehydrated in ethanol series and processed for SEM as described above.

RNA Preparation and Northern Blots

Total RNA extracts were prepared from cells plated at the initial density of $5 \times 10^3/cm^2$ into 75-cm^2 tissue flasks and cultivated in similar conditions as described above. For assay, cells were washed in PBS, pH 7.4, lysed with 6.8-mM guanidine thiocyanate and RNAs were precipitated with isopropanol (v/v) and 2-M LiCl. Aliquots of RNAs (20 μg) were fractionated on 1.2% agarose formaldehyde gels. RNAs were transferred during 16 h by capillarity to 0.2 μm nylon membranes and immobilized by baking at 80°C for 2 h.

The membranes were prehybridized and hybridized at 42°C in 5 × Denhardt's solution, 5 × SSPE (solution of sodium phosphate EDTA), 50% formamide, 250 μg/ml salmon DNA testes with [^{32}P]-labeled cDNA probes for rat ALP (rALP), rat OC (rOC), mouse BSP (mBSP) and rat GAPDH (rGAPDH). The probes were ^{32}P labeled using a DNA labeling kit. The membranes were washed twice in 2 × SSC (solution of sodium citrate) and 0.1% SDS (sodium dodecyl sulfate) at room temperature, and twice in 0.1 × SSC and 0.1% SDS at 50°C. X-Omat AR radiographic films (Kodak, Rochester, NY) were exposed to the membranes, using two intensifying screens at − 70°C.

Statistical Analysis

For assays in triplicate (proliferation, phosphatase alkaline) and cell counts, means and standard deviations (SD) were performed using Systat software release 5.2.1 (SPSS Inc. Chicago, IL). Significance were done by Student's *t*-test.

RESULTS

Proliferation

Treatment with 10^{-8} M dex significantly increased cell proliferation in subcultured rat bone marrow cells as measured by [methyl-^{3}H] thymidine incorporation. This effect appeared with similar amplitude when cultures were treated continuously (Fig. 2a) with dex (day 4, 7, 14) or discontinuously (Fig. 2b), after a 7 day period free of hormone (day 11, 14, 21). In contrast, 10^{-8} M calcitriol, alone or associated with 10^{-8} M dex, decreased cell proliferation. Cultures were stopped after 2 weeks of dex treatment because of the cell unsticking.

Calcified Nodules

In primocultures, in presence of dex, ascorbic acid and β-glycerophosphate, a large population of ALP^+ cells develops and calcified nodules were identified from day 7, but processed for analysis at day 16. Under light microscopy, the calcified formations appeared covered and surrounded by ALP^+ globular cells (Fig. 3a). Between the nodules, cells displayed a flattened

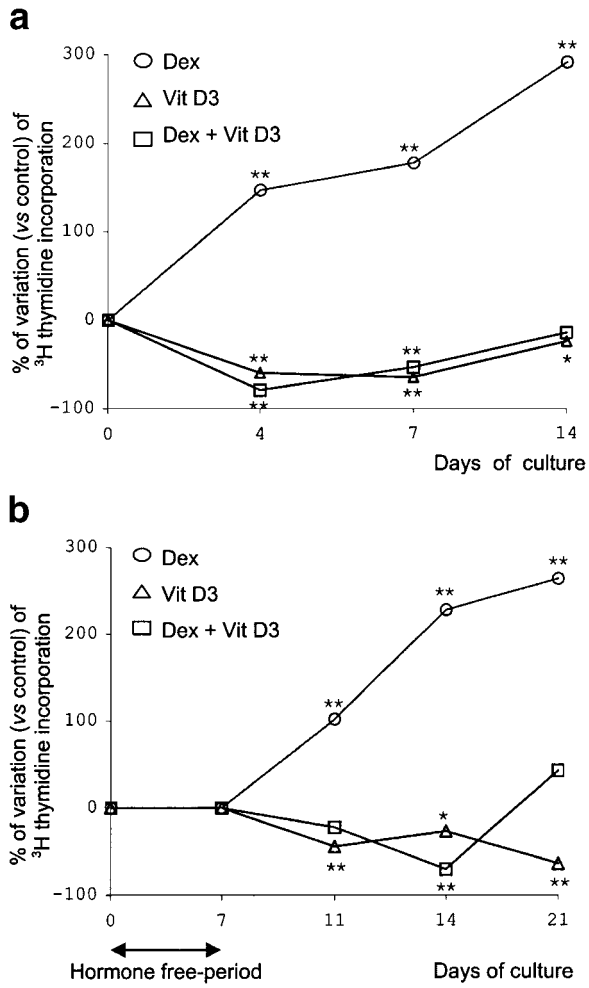

Fig. 2. Rat bone marrow stromal cells were grown in subculture. Continuous hormonal treatment (**a**) with 10^{-8} M dex increased cell proliferation, whereas 10^{-8} M calcitriol, alone or associated with 10^{-8} M dex decreased it. After an initial 7 day period free of hormonal supplementation (**b**), the recovery of hormonal treatment induced similar effects on cell proliferation. $^*P < 0.05$ vs. control, $^{**}P < 0.001$ vs. control.

appearance with numerous cytoplasmic slender processes. In SEM, a dense fibrillar matrix (fibrils 40–80 nm in diameter) was observed, associated with the cell layers (Fig. 3b). These fibrils were identified as type I collagen fibrils by immuno-gold detection (Fig. 3c). Mineral deposits appeared as conglomerates entangled with collagen fibrils (Fig. 3b). They were identified with backscattered electrons and X-ray electron microanalysis indicated a 1.68 ± 0.09 Ca/P ratio (Fig. 3d).

Histochemical Staining of ALP

With continuous treatment by dex (Fig. 4a), the number of ALP^+ cells markedly increased vs. control ($+75.9\% \pm 15\%$ at D14, $P < 0.001$), whereas calcitriol decreased ALP^+ cell number ($-78.1\% \pm 1.6\%$ at D14, $P < 0.001$) and stopped the dex upregulation ($-94.3\% \pm 2.4\%$ at D14, $P < 0.001$).

When subcultures were performed with a 7-day gap of hormone treatment (Fig. 4b), the number of ALP^+ cells decreased even after recovery of dex or calcitriol treatment. At D21 (i.e., 14 days after hormonal treatment recovery), the number of ALP^+ cells decreased in all conditions vs. control ($-85.7\% \pm 6.02\%$ with dex, $P < 0.001$, $-81.4\% \pm 3.5\%$ with calcitriol, $P < 0.001$, and $-90.5\% \pm 4.8\%$ with both hormones, $P < 0.001$).

ALP Bioactivity

ALP bioactivity was strongly enhanced in subcultures continuously treated by 10^{-8} M dex vs. control ($+638$ % at day 14, $P < 0.001$) (Fig. 5a). Calcitriol, alone or associated with dex, decreased the ALP enzymatic activity (respectively -76.1% and -61.9% at day 14, $P < 0.001$). After the free hormonal period (Fig. 5b), recovery of dex, or calcitriol, or both, induced a significant decrease ($P < 0.001$) of ALP activity vs. control condition (respectively -49.3, -59.7, and -63.3% at D21, i.e. after 14 days of hormonal recovery treatment).

Expression of mRNAs

After 7 days of primoculture, ALP, OC, and BSP mRNAs were strongly expressed (Fig. 6). In subcultures, dex strongly induced expression of OC and BSP, with a maximal expression at day 7 (Fig. 7). No effect on ALP mRNA could be observed. In contrast, calcitriol strongly decreased ALP mRNA and slightly increased OC mRNA expression, but did not induce expression of BSP mRNA. Furthermore, calcitriol increased the stimulating effect of dex on OC mRNA expression, but decreased the stimulating effect of dex on BSP mRNA.

When subcultures were performed after an initial hormone-free period (Fig. 8), ALP mRNA expression was decreased by dex, calcitriol, and both hormones; OC mRNA was not induced by either of the hormones, but their association slightly induced OC mRNA expression; BSP mRNA was slightly induced by dex, and this effect was decreased by calcitriol.

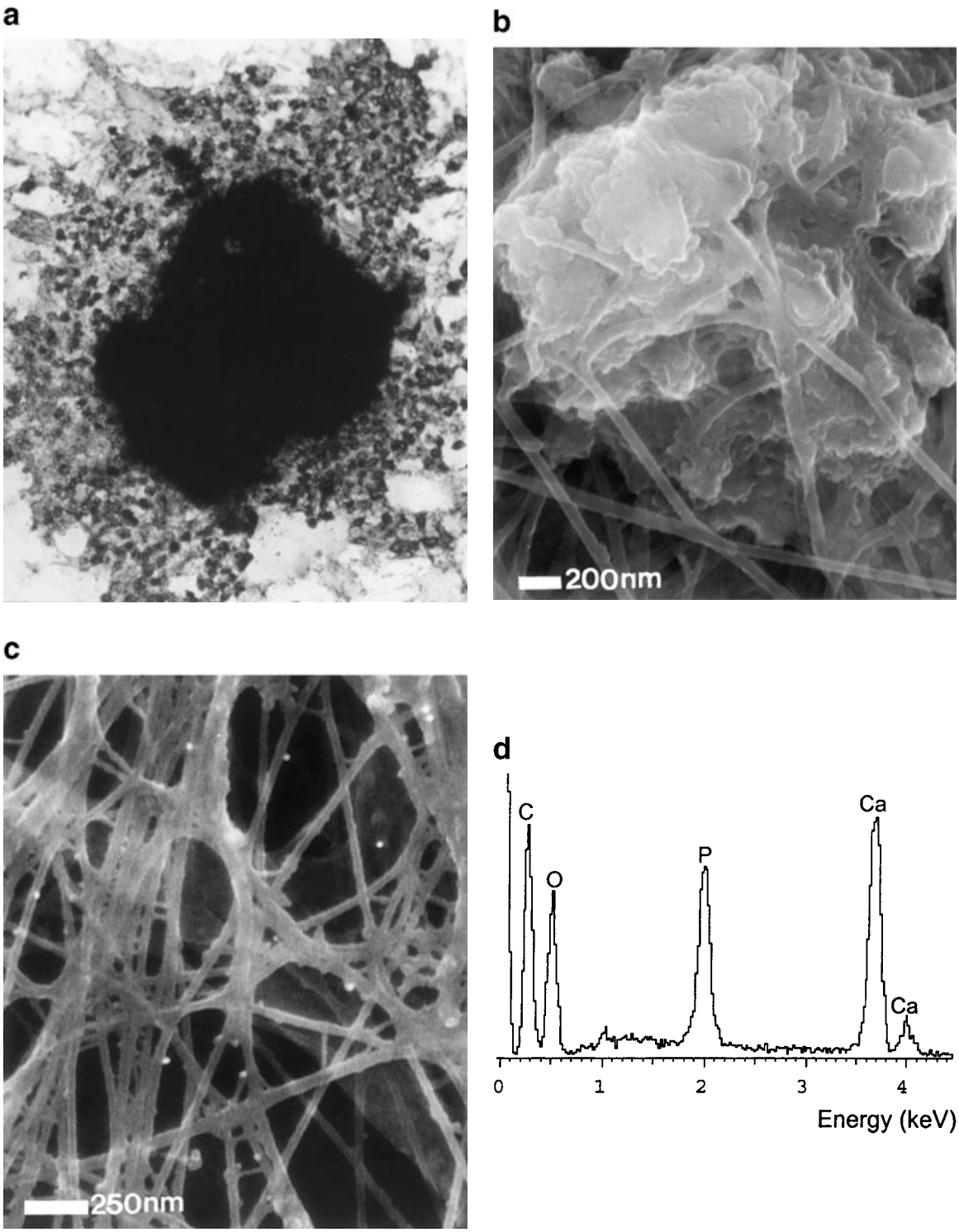

Fig. 3. Rat bone marrow stromal cells were grown in primoculture in presence of 10^{-8} M dex and 10 mM β-glycerophosphate. At day 16, numerous calcified nodules were identified (Von Kossa) associated with ALP^+ cells (**a**). In SEM, the calcified nodules appeared made of microfibrils entangled with mineral deposits (**b**). These microfibrils were identified by immunogold detection in SEM as type I collagen (**c**). The Ca/P ratio (1.68 ± 0.09) of the calcified nodule was determined by EDX (**d**).

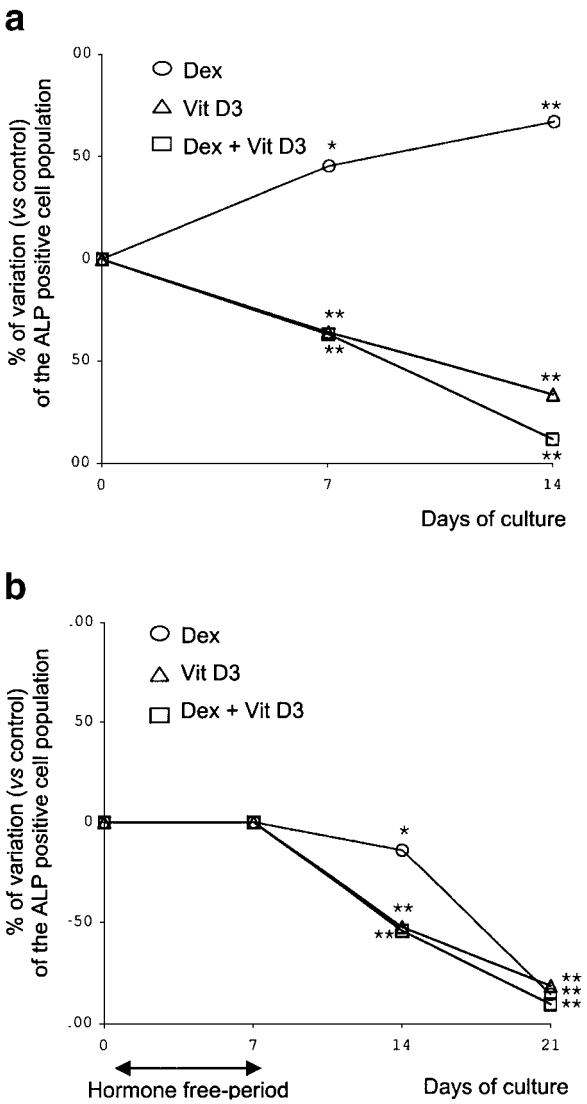

Fig. 4. Rat bone marrow stromal cells were grown in subculture. Continuous treatment (**a**) with 10^{-8} M dex increased ALP^+ cell population, whereas 10^{-8} M calcitriol, alone or associated with 10^{-8} M dex decreased it. After the 7 day period free of hormonal treatment, recovery of dex or calcitriol or both decreased the ALP^+ cell population (**b**). $^*P < 0.05$ vs. control, $^{**}P < 0.001$ vs. control.

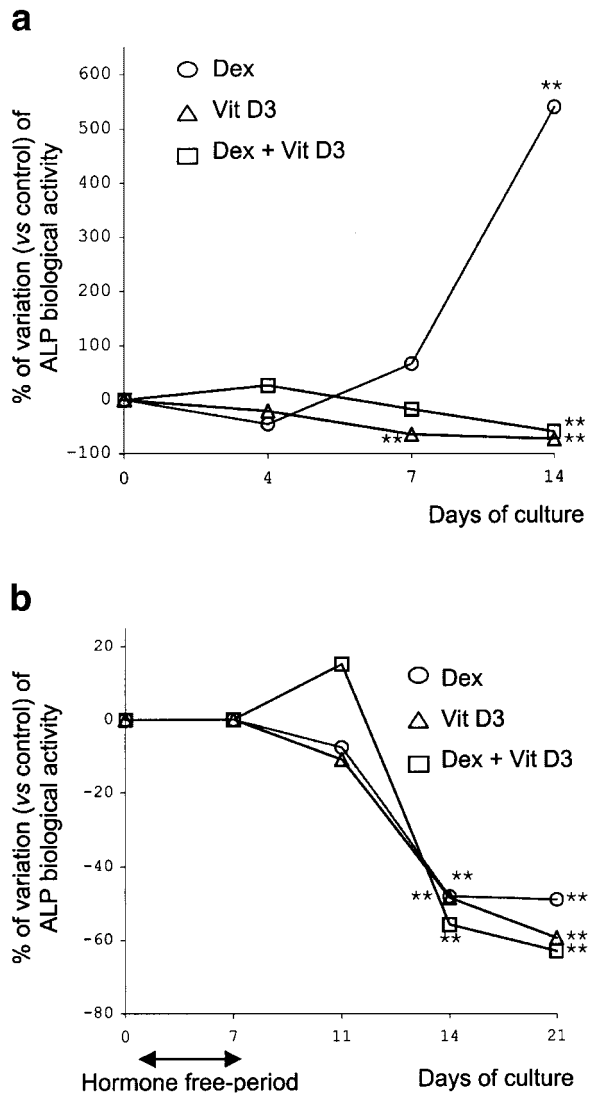

Fig. 5. Continuous treatment of rat bone marrow stromal cells grown in subculture with 10^{-8} M dex (**a**) strongly increased ALP biological activity, whereas 10^{-8} M calcitriol, alone or associated with 10^{-8} M dex decreased it. The initial 7 day period free of hormonal supplementation (**b**) induced a significant decrease of ALP biological activity in subculture even after recovery of hormonal treatment. $^{**}P < 0.001$ vs. control.

DISCUSSION

Many factors have been identified to promote in vitro the differentiation of osteoblasts and dex, a synthetic glucocorticoid, was reported as one of the most efficient [Maniatopoulos et al., 1988; Malaval et al., 1994; Aubin, 1999]. A large osteoblastic cell population can be raised in bone marrow cell primocultures supplemented with dex, L-ascorbic acid (requested for collagen production and ALP activity enhancement) [Choong et al., 1993], and β-glycerophosphate (acting as a substrate for ALP to allow mineralization) [Peter et al., 1998].

The osteoblasts obtained in this work, using similar culture conditions, were mature and functional. In primocultures, they strongly expressed ALP, OC, and BSP mRNAs; they synthesized type I collagen and formed calcified nodules with a ratio Ca/P similar to that found in natural bone. Cells expressing ALP were present with a great density in calcified nodules.

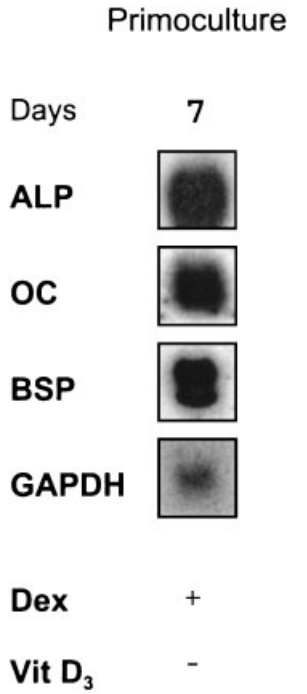

Fig. 6. Rat bone marrow stromal cells mRNAs were studied by Northern blot after 7 days of primoculture in presence of 10^{-8} M dex. The expression of ALP, OC, and BSP mRNAs was strongly evidenced.

These data support that the primocultures contained a high density of mature and active osteoblasts.

When the rat bone marrow-derived cells were subcultivated in continuous presence of dex, cell proliferation was stimulated and the osteoblastic phenotype was induced. High ALP activity and high expression of different osteoblastic markers like ALP, BSP, and OC were observed. Moreover, ALP bioactivity was maintained at a high level at day 14 of culture, whereas ALP, BSP, and OC mRNA expression decreased. These findings are consistent with the decrease at day 12 of ALP and BSP mRNAs and the decrease after day 15 of OC synthesis, observed in dex-treated cultures of bone marrow cells from rat femurs [Malaval et al., 1994]. Similar results were also observed with chronically dex-treated osteoblasts isolated from fetal rat calvaria [Shalhoub et al., 1998]. These findings were proposed to be the result of an accelerated differentiation and consistent with reports showing, in primary osteoblasts, the decrease of OC expression after day 28 [Stein and Lian, 1993; Shalhoub et al., 1994].

In contrast, calcitriol reduced cell proliferation and inhibited the mRNA expression and the activity of ALP. Calcitriol also did not induced BSP expression, but slightly increased the OC messengers level. Moreover, in presence of calcitriol, the dex-increase of cell proliferation and ALP bioactivity, the dex-induced BSP expression, and the dex-level of the ALP expression were reduced. Conversely, calcitriol strongly enhanced the increasing effect of dex on OC mRNA levels.

These results are in agreement with previous reports. It was shown that dex increases ALP-positive cell number and/or ALP bioactivity in rat bone marrow stromal cells [Ohgushi et al., 1996; Peter et al., 1998], in human bone marrow stromal cells [Fromingué et al., 1997], and in osteoblast-like cells isolated from human bone biopsies [Kasperk et al., 1995]. It was also described that dex increases the steady state of BSP mRNA, whereas calcitriol inhibited this effect in ROS 17/2.8 cells [Oldberg et al., 1989]. OC expression is known to be increased by dex and by calcitriol in rat bone marrow stromal cells [Ohgushi et al., 1996; Van Leeuwen et al., 1996; Peter et al., 1998], in human osteosarcoma cells [Van Leeuwen et al., 1996] and in human bone marrow cells [Liu et al., 1999; Shibano et al., 1998]. Others researchers have shown that in rat osteoblastic osteosarcoma cells [Majeska and Rodan, 1982] and in human bone marrow stromal cells [Shibano et al., 1998], calcitriol stimulates ALP bioactivity, but can also inhibit differentiation when added continuously to proliferating osteoprogenitor cells [Lian and Stein, 1993].

These data confirm that dex is a major factor for the expression of osteoblastic phenotype markers like BSP, OC, and ALP messengers or ALP activity. Calcitriol seems to have a dissociated effect on this phenotype because it markedly decreased the dex effect on ALP and BSP mRNA expression and on ALP biological activity, while it induced OC expression and enhanced the stimulating effect of dex on this marker.

When rat bone marrow cells were initially subcultivated in hormone-free conditions, the recovery of dex supplementation stimulated the cell proliferation, whereas calcitriol inhibited it, even in association with dex. The reintroduction of dex after the hormone-free period, reduced expression of BSP and ALP messengers in regard to levels obtained with continuous treat-

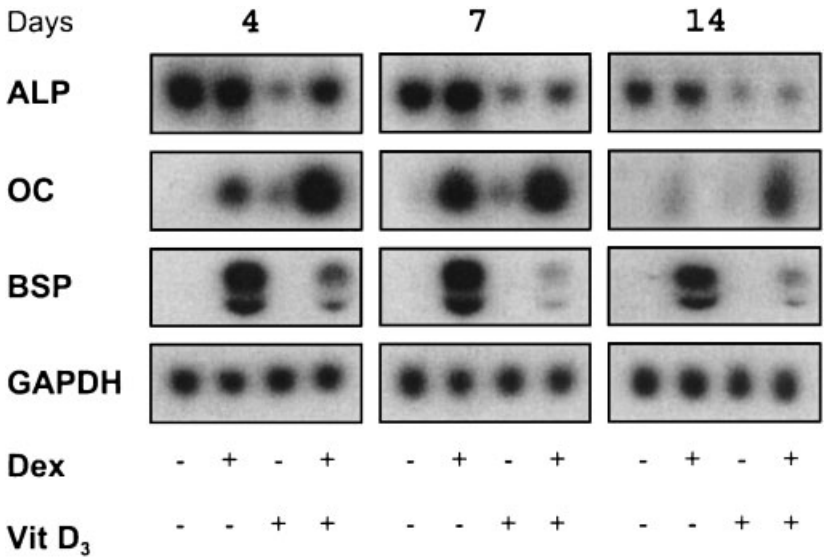

Fig. 7. Rat bone marrow stromal cells were grown in primoculture in presence of 10^{-8} M dex and then subcultured for 4, 7, and 14 days in presence of 10^{-8} M dex, or 10^{-8} M vitamin D_3 or both hormones. Dex induced OC and BSP mRNA expression, calcitriol decreased ALP mRNA expression and slightly induced OC expression, and both hormones enhanced OC mRNA expression.

ment. OC messengers were not detected with recovery of dex treatment alone and were found strongly decreased with the combination dex-calcitriol.

Recent data suggest that in rat bone marrow there are at least two subpopulations of osteoblastic precursors depending on their request in glucocorticoids to differentiate [Aubin, 1999]. The subpopulation, which does not require dex to differentiate, was supposed to be in minority. When cultured in absence of dex during 7 days, the population that needs glucocorticoids to differentiate, is not stimulated and lose its capacity to differentiate into osteoblasts even after recovery of dex treatment. Conversely, the population that does not require glucocorticoids to differentiate, subsisted after the hormone-free period, but their small amount could be responsible for the low level of the expression of osteoblastic markers.

These results are conflicting with a recent report demonstrating that cortisol decreased

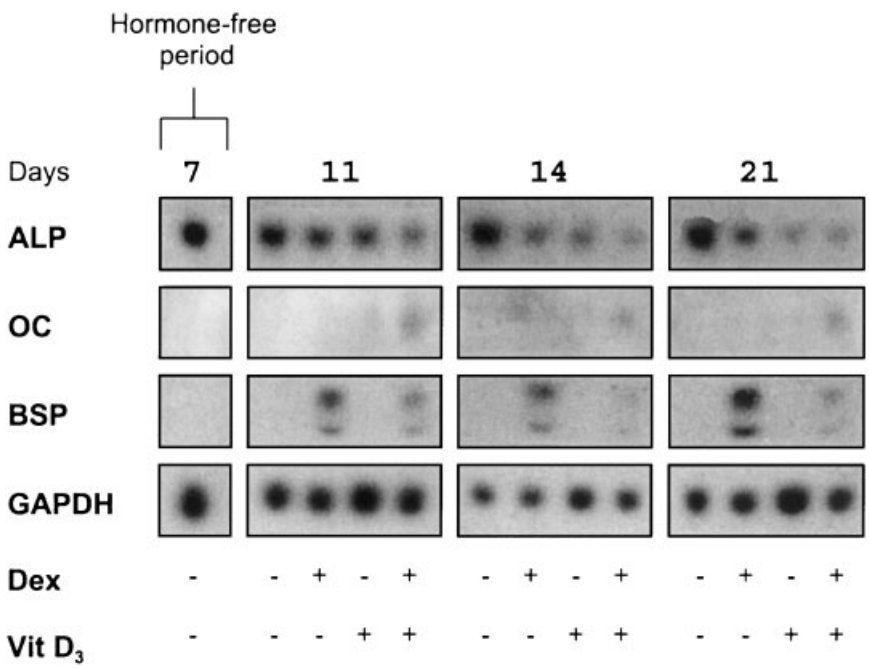

Fig. 8. Rat bone marrow stromal cells were subcultured for 7 days without hormonal supplementation. Recovery of 10^{-8} M dex or 10^{-8} M vitamin D_3 or both hormones strongly decreased the expression of osteoblast-associated marker mRNAs (compare to Fig. 7).

cell proliferation and inhibited the differentiation of osteoblastic cells from fetal rat calvariae [Pereira et al., 2001]. However, the conditions of culture were not similar in our study. In the above report, cells were obtained from fetal rat parietal bone. Responses of osteoblasts to hormones or various growth factors were found different according to the species [Bellows et al., 1998; Chen and Fry, 1999; Thomas et al., 2000] or sexes [Ishida and Heersche, 1997]. Even in the same bone, various classes of osteogenic precursors have been supposed [Aubin, 1999]. Behavior of osteoblasts from femur or parietal bones could be different because femur results of endochondral ossification, whereas parietal bone develops from intramembranous ossification. Moreover, the glucocorticoids used (cortisol, dex) have different binding affinity with the glucocorticoid receptor and biological potency [Ishida and Heersche, 1998]. So, the class of the glucocorticoid used and its concentration in culture could also explain variations in the type and level of the osteoblastic responses. With 10^{-8} M dex, osteoblastic phenotype is stimulated in male rat bone marrow cell cultures, whereas 10^{-6} M cortisol could inhibit the differentiation of parietal bone osteoblasts [Ohgushi et al., 1996; Peter et al., 1998; Aubin, 1999; Pereira et al., 2001].

Glucocorticoid therapy is known, for a long time, to induce the development of osteoporosis and chronic administration of glucocorticoids, at therapeutic concentration, induces apoptosis in osteoblast, decreases the osteoblast precursors population and mature osteoblast number, and reduces bone formation [Manolagas and Weinstein, 1999]. Conversely, glucocorticoids at concentrations within physiological range promote the recruitment, the differentiation and the maturation of osteoblasts [Beresford et al., 1994; Jaiswal et al., 1997; Walsh et al., 2001]. Our data suggest that continuous presence of glucocorticoids appears to be required to support the permanent expression of the osteoblastic phenotype.

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