A Newly Established Cell Line from Normal Human Bone Responds to 1α, 25-Dihydroxyvitamin D_3, Retinoic Acid and Transforming Growth Factor-β_1

Hideki CHIBA,[1,2] Norimasa SAWADA,[1] Kousuke IBA,[1,2]
Hiroshi ISOMURA,[1] Seiichi ISHII[2] and Michio MORI[1]

Departments of [1]Pathology and [2]Orthopedic Surgery,
Sapporo Medical University School of Medicine, S. 1, W. 17, Chuo-ku, Sapporo 060, Japan

ABSTRACT

We have recently established a new osteoblastic cell line, designated SV-HFO, from normal human bone by immortalization with simian virus 40. In the present study, we examined the effects of diffusive factors on the expression of osteoblastic phenotype in SV-HFO cell line. 1α, 25-dihydroxyvitamin D_3 induced the expression of alkaline phosphatase (ALP) and osteocalcin. Retinoic acid down-regulated the expression of ALP, whereas it up-regulated the expression of osteocalcin. Transforming growth factor-β_1 reduced the expression of both osteoblastic properties. These effects were time- and dose-dependent. These results show that the SV-HFO cell line maintains responsiveness to these diffusive factors. This cell line is suitable model for studying both metabolism and multistep carcinogenesis of human bone.

Key words: Human osteoblasts, Simian virus 40, Cell line, Differentiation

INTRODUCTION

Several cell lines derived from human osteosarcoma tissues have been extensively employed for study of human osteosarcomas *in vitro* (1-4), while normal human osteoblasts in culture are generally used for study of the metabolism of human bone (5-8). However, the phenotypic properties of normal human osteoblasts tend to deteriorate with increasing passage number, and the cells eventually lose their proliferative activity. Thus, immortalized osteoblastic cell lines from normal human bone have long been desired for studying the metabolism and multistep carcinogenesis of human bone.

We have recently established a human osteoblastic cell line from normal fetal bone by immortalization with simian virus 40. This cell line, designated SV-

HFO, has proliferated and has maintained its osteoblastic features even under serum-free conditions as well as under serum-supplemented conditions. These cells grew in soft agar, but did not form tumors when transplanted into athymic nude mice (9, 10). In this sense, the SV-HFO cells show intermediate properties between normal and neoplastic cells.

Transformed cells often respond abnormally to hormones or growth factors. In the present study, we examined whether the SV-HFO cells responded to major modulators of bone cells including 1α, 25-dihydroxyvitamin D_3 (1, $25(OH)_2D_3$), retinoic acid and transforming growth factor-β (TGF-β).

MATERIALS AND METHODS

Cell culture

The SV-HFO cells were established as described previously (9). The cells at passage 15 were seeded at a cell density of 1×10^4 calls/cm^2 on 100-mm culture dishes or 12-well tissue culture plates coated with 2 μg/cm^2 type I collagen (Vitrogen 100; Collagen Corp., Palo Alto, CA) in serum-free Dulbecco's modified Eable's medium (DMEM; Nissui Pharmaceutical, Tokyo, Japan) supplemented with 0.5% bovine serum albumin (Albumax; Gibco Laboratories, Grant Island, NY), ITS (containing 5 μg/m*l* insulin, 5 μg/m*l* transferrin and 5 ng/m*l* selenious acid; Collaborative Research, Inc., Bedford, MA), 100 U/ml penicillin and 100 μg/m*l* streptomycin (Gibco Laboratories). These cells were cultured at 37℃ in a humidified atmosphere of 5% CO_2 in air, and the medium was renewed every 3 days. To determine the effects of 1, $25(OH)_2D_3$ (Biomol Research Laboratories, Inc., Plymouth Meeting, PA), all-trans retinoic acid (RA) or TGF-β_1 (Sigma Chemical Co., St. Louis, MO) on the synthesis of ALP and osteocalcin, the cells reaching a confluent density on 12-well tissue culture plates were treated with the modulators at various concentrations for 3 consecutive days. To examine the effects of 1, $25(OH)_2D_3$, RA or TGF-β_1 on the expression of ALP and osteocalcin mRNA, the cells reaching a confluent density on 100-mm culture dishes were treated with 10^{-7}M 1, $25(OH)_2D_3$, 10^{-7}M RA or 5 ng/m*l* TGF-β_1 for 6, 12, 24 or 48 consecutive hours. The stock solution of 1, $25(OH)_2D_3$ was in ethanol, and that of RA was in dimethyl sulfoxide (DMSO). Both test and control solutions contained the same concentration of ethanol or DMSO, which was less than 0.1%.

Measurement of ALP activity

After treatment with modulators, the cells were rinsed twice with PBS, scraped at 37℃ in 0.3 m*l* of a solution containing 0.1% Triton X-100, 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$ and 0.02% NaN_3, and sonicated for 5 sec.

The cell extracts were centrifuged for 5 min at 10,000 g at 4℃, and kept frozen at −20℃ until use.

ALP activity was spectrophotometrically measured using p-nitrophenyl-phosphate as a substrate according to the method of Lowry (11). The content of cellular protein was measured by the Pierce BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA) using BSA as a standard. ALP activity was expressed as nanomoles/minute/milligram protein.

Measurement of osteocalcin synthesis

The amount of osteocalcin secreted into the culture medium was determined by radioimmunoassay using a commercially available kit (CIS Biointernational, Gif-Sur-Yvette, France). Results were expressed as nanograms/10^6 cells.

Northern blot analysis

Total RNAs were isolated from cell cultures using the single step thiocyanate-phenol-chloroform extraction method (12) as modified by Xie and Rothblum (13). For electrophoresis, 10 μg of total RNAs was loaded on 1% agarose gel containing 0.5 μg/m*l* ethidium bromide. Gels were capillary-blotted in 20× saline sodium citrate (SSC) onto nylon membranes (Hybond-N; Amersham Corp., Buckinghamshire, England) and fixed by UV light.

For the detection of ALP mRNA, membranes were prehybridized in a solution containing 50% formamide, 0.9 M NaCl, 0.1 M $NaPO_4$ (pH 7.4), 1% sodium dodecyl sulfate (SDS), 10 μg/m*l* herring sperm DNA and 5× Denhart's solution for 4h at 42℃, and then hybridized overnight at 42℃ in the same solution with a ^{32}P-labeled cDNA probe for human ALP obtained from the American Type Culture Collection. Next, the membranes were washed twice in 2× SSC buffer containing 0.1% SDS for 5 min at room temperature and twice in 2× SSC buffer containing 1% SDS for 30 min at 68℃ before exposure to film.

To identify osteocalcin mRNA, digoxigenin (DIG)-labeled RNA probe were prepared from rat cDNA using an RNA labeling kit (Boehringer Mannheim, Mannheim, Germany), and hybridization, washing and chemiluminescent detection were carried out following the DIG luminescent protocol (14).

RESULTS

Only cells reaching a confluent density were used to minimize the influence of cell proliferation on the expression of ALP and osteocalcin. In addition, to exclude the effect of serum on the expression of above mentioned proteins, the cells were cultured under the serum-free condition.

Effects of 1, 25(OH)$_2$D$_3$, RA and TGF-β_1 on ALP activity

Figure 1 shows the effects of 1, 25(OH)$_2$D$_3$ and RA on ALP activity in SV-HFO cells. Treatment of cells with 1, 25(OH)$_2$D$_3$ at concentrations higher than 10^{-9}M for 3 days significantly induced ALP activity, with the maximal effect (3.7-fold increase) at 10^{-7}M. In contrast, RA lowered ALP activity at concentrations higher than 10^{-9}M. After treating cells with 10^{-6}M RA for 3 days, the ALP activity was reduced to approximately half of the control. Addition of both 10^{-7}M 1, 25(OH)$_2$D$_3$ and 10^{-7}M RA resulted in a slight increase in ALP activity, but the level was significantly lower than that induced by 10^{-7}M 1, 25(OH)$_2$D$_3$, TGF-β_1 significantly reduced ALP activity (Table I).

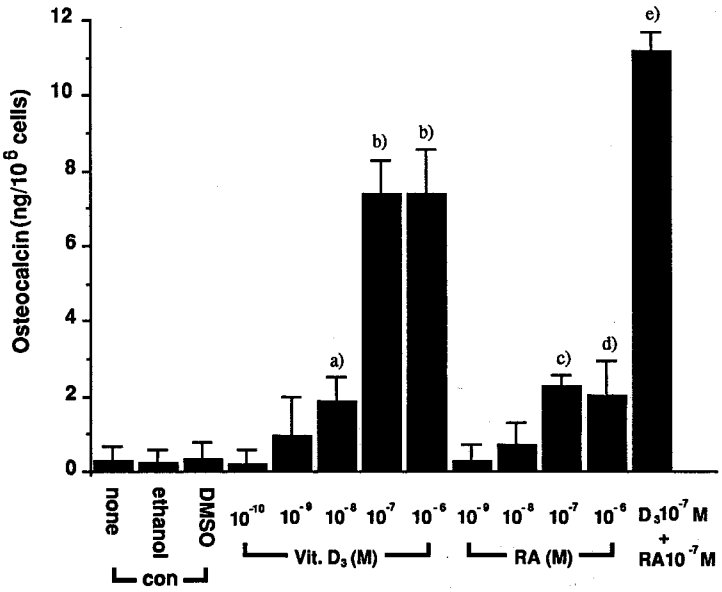

Fig. 1 Effects of 1, 25(OH)$_2$D$_3$ and RA on alkaline phosphatase activity in the SV-HFO cells after they reached confluency. The figures are mean values ± SD of four different wells with duplicate determinations. a), b) Significantly different from the value of the control cells cultured with 0.1% ethanol and without 1, 25(OH)$_2$D$_3$ (a) $P<0.01$, b) $P<0.001$). c), d) Significantly different from the value of the control cells cultured with 0.1% DMSO and without RA (c) $P<0.05$, d) $P<0.01$).

Effects of 1, 25(OH)$_2$D$_3$, RA and TGF-β_1 on osteocalcin production

Treatment of the cells with 1, 25(OH)$_2$D$_3$ or RA increased the amount of osteocalcin in the medium in a dose-dependent manner. The maximal effects of 1, 25(OH)$_2$D$_3$ and RA (28.5-fold and 6.5-fold, respectively) were seen at 10^{-7}M. 1, 25(OH)$_2$D$_3$ and RA appeared to be synergistic in action in osteocalcin produc-

Table I. *Effects of TGF-β_1 on alkaline phosphatase activity and the amount of osteocalcin in the SV-HFO cells after they reached confluency.*

Treatment	Alkaline phosphatase activity (nmol/min/mg protein)	Osteocalcin (ng/10^6cells)
None	36.14±2.79	0.32±0.12
TGF-β_1 0.05 ng/m*l*	36.14±1.82	0.36±0.13
0.5 ng/m*l*	30.95±2.58[a)]	0.27±0.08
5 ng/m*l*	25.73±3.12[b)]	0.06±0.07[a)]

The figures are mean values ± SD of four different wells with duplicate determinations. a), b) Significantly different from the value of the control cells cultured without TGF-β_1 (a) $P<0.05$, b) $P<0.01$).

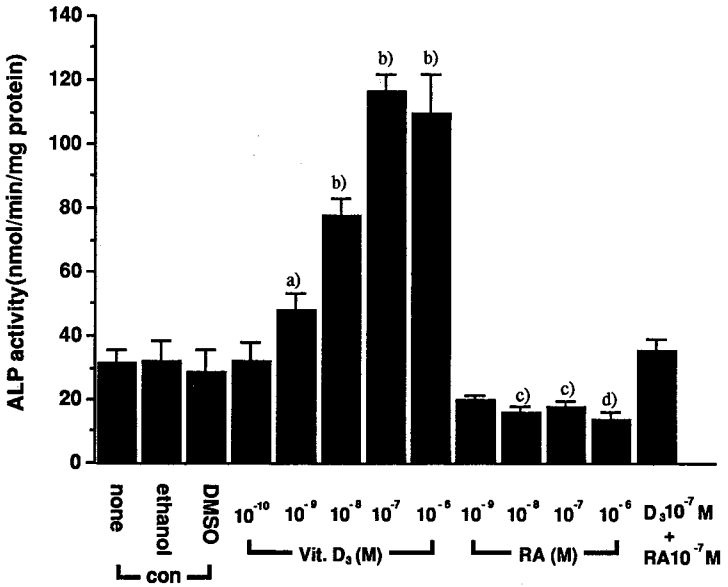

Fig. 2 Effects of 1, 25$(OH)_2D_3$ and RA on the amount of osteocalcin in the SV-HFO cells after they reached confluency. The figures are mean values ± SD of four different wells with duplicate determinations. a), b) Significantly different from the value of the control cells cultured with 0.1% ethanol and without 1, 25$(OH)_2D_3$ (a) $P<0.01$, b) $P<0.001$). c), d) Significantly different from the value of the control cells cultured with 0.1% DMSO and without RA (c) $P<0.001$, d) $P<0.05$). e) Significantly different from the value of the control cells cultured with 0.1% ethanol or 0.1% DMSO and without 1, 25$(OH)_2D_3$ and RA ($P<0.001$).

tion (Fig. 2). TGF-β_1 significantly suppressed osteocalcin synthesis at 5 ng/m*l* (Table I). TGF-β_1 also reduced 1, 25$(OH)_2D_3$- or RA-induced osteocalcin pro-

duction (data not shown).

Effects of 1, 25(OH)$_2$D$_3$, RA and TGF-β_1 on the expression of ALP and osteocalcin mRNA

We further examined the changes in the expression of ALP and osteocalcin mRNA in SV-HFO cells treated with 10^{-7}M 1, 25(OH)$_2$D$_3$, 10^{-7}M RA or 5 ng/m*l* TGF-β_1 (Figs. 3 and 4).

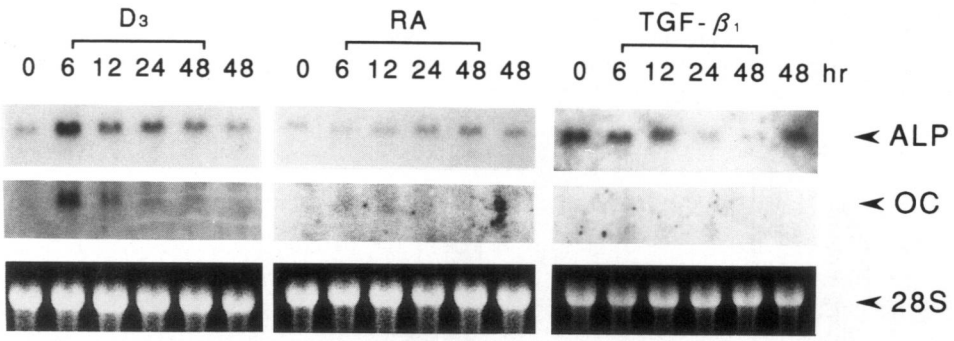

Fig. 3 Northern blot analysis of the transcripts of genes of ALP and osteocalcin after treating confluent SV-HFO cells with 10^{-7}M 1, 25(OH)$_2$D$_3$, 10^{-7}M RA or 5 ng/m*l* TGF-β_1. The bottom panel shows ethidium bromide stain of the filter corresponding to 28S ribosomal RNA.

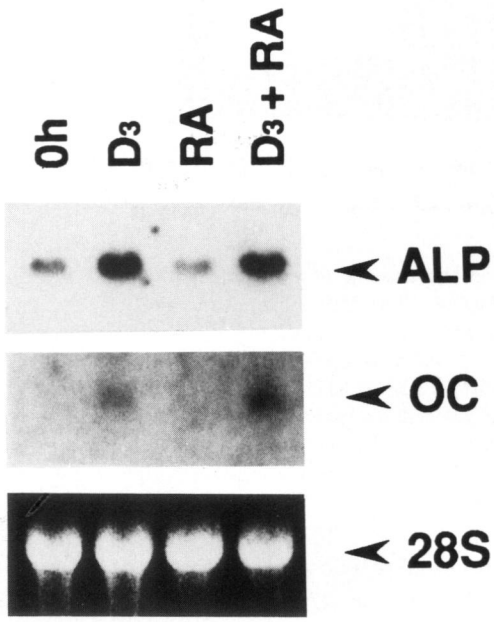

Fig. 4 Northern blot analysis of the transcripts of genes of ALP and osteocalcin 6 hours after treating confluent SV-HFO cells with 10^{-7}M 1, 25(OH)$_2$D$_3$, 10^{-7}M RA, or a combination of the two. The bottom panel shows ethidium bromide stain of the filter corresponding to 28S ribosomal RNA.

The expression of ALP mRNA was up-regulated by 10^{-7}M 1, 25$(OH)_2D_3$ in a time-dependent manner for at least 48 hours, with a maximal increase at 6 hours. In contrast, the expression was down-regulated by 10^{-7}M RA, with the lowest value at 6 hours. The expression was also decreased by treating the cells with 5 ng/m*l* TGF-β_1 for 24 to 48 hours.

The expression of osteocalcin mRNA was significantly increased by treating the cells with 10^{-7}M 1, 25$(OH)_2D_3$ or 10^{-7}M RA, which reached the maximum at 6 hours after treatment. The effect of 1, 25$(OH)_2D_3$ was much greater than that of RA, and both modulators acted in a synergistic manner to osteocalcin expression. On the other hand, TGF-β_1 did not enhance the expression.
1, 25$(OH)_2D_3$, RA or TGF-β_1 modulated the expression of ALP and osteocalcin mRNA in a dose-dependent manner (data not shown).

DISCUSSION

We have recently established a cell line from normal human bone which possesses osteoblastic properties. This cell line, designated SV-HFO, was immunocytochemically positive for vimentin but negative for keratin and epithelial membrane antigen, which suggested that it was of mesenchymal origin. Phase-contrast microscopic and electron microscopic observations supported the mesenchymal nature of these cells. Osteoblastic nature was indicated by evidence showing that the cells produced ALP and osteocalcin (9). ALP activity in SV-HFO cells increased with increase in cell density. Osteocalcin, a bone specific protein produced by mature osteoblasts (15, 16), was not induced in the cells at sparse or subconfluent density, but became detectable in the cells after they reached confluency (10).

In the present experiments, we further examined the responsiveness of SV-HFO cells to 1, 25$(OH)_2D_3$, RA and TGF-β_1 using ALP and osteocalcin as a marker. To minimize the influence of cell proliferation, we used the cells only after they had reached a confluent cell density. The expression of ALP in SV-HFO cells was enhanced by 1, 25$(OH)_2D_3$, but suppressed by either RA or TGF-β_1. It was also shown that the expression of osteocalcin was up-regulated by 1, 25$(OH)_2D_3$ or RA, but down-regulated by TGF-β_1. These effects were dose- and time-dependent.

Reduced expression of ALP by RA treatment was also observed in cultured normal human osteoblastic cells (17; Chiba *et al.*, unpublished data). These findings are inconsistent with some previous reports which showed that RA treatment induces ALP expression in rodent osteoblast-like and undifferentiated mesenchymal cells (18-21). To achieve function, steroids and related hormones must bind to their receptors complex to responsive elements in target genes (22-

26). Although the mechanism of transcriptional repression by steroids hormones remains obscure, Demay et al. have recently reported that there is a down-regulatory 1, $25(OH)_2D_3$ responsive element which differs from the up-regulatory one, both in sequence composition and in its requirement for particular cellular factors (27). From this viewpoint, our observations seem to mean that the human ALP gene has the down-regulatory RA responsive element, while the rodent ALP gene contains the up-regulatory one.

The inhibitory effect of TGF-β_1 on ALP expression is consistent with the results obtained from primary culture and cell lines derived from "normal bones" of rodents (28-32), but not with those observed in cell lines derived from rat and human "osteosarcomas" (33-35). Thus, SV-HFO cells are considered to maintain normal responsiveness to 1, $25(OH)_2D_3$, RA and TGF-β_1 at least the expression of ALP and osteocalcin is concerned. We have also observed that the cells have a potential to form mineralized tissues *in vitro*, which suppressed by RA or TGF-β_1 (in preparation). These results show that this cell line is a new model for elucidating not only multistep carcinogenesis but also metabolism of human bone.

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