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Prolonged Culture of HOS 58 Human Osteosarcoma Cells With 1,25-$(OH)_2$-D_3, TGF-Beta, and Dexamethasone Reveals Physiological Regulation of Alkaline Phosphatase, Dissociated Osteocalcin Gene Expression, and Protein Synthesis and Lack of Mineralization

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Abstract Cultured rodent osteoblastic cells reiterate the phenotypic differentiation and maturation of osteoblasts seen in vivo. As previously shown, the human osteosarcoma cell line HOS 58 represents a differentiated stage of osteoblast development. The potential of HOS 58 for still further in vitro differentiation suggests the line can serve as a model of osteoblast maturation. Using this cell line, we have investigated the influence of 1,25-$(OH)_2$-D_3 (D_3), TGF-beta and Dexamethasone (Dex) on proliferation and on the protein and mRNA levels of alkaline phosphatase (AP), procollagen 1 (Col 1), and osteocalcin (Oc), as well as mineralization during 28 days in culture. AP mRNA and protein were highly expressed throughout the culture period with further increase of protein AP activity at constant gene expression levels. A differentiation inhibiting effect of either TGF-beta or Dex was seen. Col 1 was investigated without the use of ascorbic acid and showed only minor changes during culture time or stimulation. The gene expression for Oc increased continually whereas protein synthesis peaked at confluence and decreased thereafter. TGF-beta and Dex treatments decreased Oc mRNA and protein levels. Stimulation by D3 was maximal at day 7 with a decrease thereafter. HOS 58 cells showed no mineralization capacity when stimulated with different agents, as measured by energy-dispersive X-ray microanalysis. This was not due to absence of Cbfa1 expression. In conclusion, the HOS 58 osteosarcoma cell line represents a differentiated cell line with highly expressed and physiologically regulated AP expression during further differentiation in culture. We observed a dissociation between osteocalcin gene expression and protein secretion which may contribute to the lack of mineralization in this cell line. J. Cell. Biochem. 85: 279–294, 2002.

Key words: osteosarcoma cells; osteoblast differentiation; mineralization; osteocalcin; alkaline phosphatase; collagen I; TGF-beta; dexamethasone; vitamin D

Rat calvarial osteoblasts maintained long-term in vitro [Owen et al., 1990; Stein et al., 1990] show maturation sequences comparable to those observed in vivo in neonatal bones [Weinreb et al., 1990; Jingushi et al., 1992; Suva et al., 1993]. The developmental stages are reiterated in vitro as a linear sequence of growth- and differentiation-dependent gene activation [Stein et al., 1990]. Although this is an important model for in vitro studies of bone metabolism, the direct applicability of the system to human tissues has, to our knowledge, not been established. It is generally assumed that primary human osteoblasts differentiate in a similar fashion to the rodent models, but this has only been partly reproduced in human calvarial cell [de Pollak et al., 1997] and primary

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iliac crest bone cell cultures [Siggelkow et al., 1999a]. Differences between the species may be partly explained by the variability of primary cells from different biopsy sites and possibly by methodological differences due to the generally low cell yield in pHOB. Human immortalized osteoblast cell lines and osteosarcoma cells have inherently less methodological problems and have been effectively used to analyze certain aspects of differentiation of human osteoblasts [Marie, 1994; Spelsberg et al., 1995]. Because of the transformed nature of osteosarcoma cells, however, a deregulation of the tightly coupled relationship between proliferation and progressive expression of genes associated with bone cell differentiation has been suggested [Stein et al., 1990]. However, the transformed nature per se does not exclude the expression of certain important aspects of the osteoblastic phenotype in vitro. Indeed, many of the biological features first identified using human osteosarcoma cells have subsequently been confirmed in primary cell cultures.

We have previously investigated the in vitro differentiation potential of HOS 58 human osteosarcoma cells [Siggelkow et al., 1998]. This cell line expresses a differentiated phenotype and continues phenoptypic maturation for at least 14 days in culture. The developmental sequence exhibited by HOS 58 cells was largely comparable to that described for primary rat osteoblasts [Owen et al., 1990], but no proper mineralization of the collagenous matrix was detected by electron microscopy. It was not clear if these differences to normal bone cells are attributable to the transformed nature of the cells, or if they represent species differences between rat and man. Maturating rat calvarial osteoblasts have been shown to exhibit different responses to stimulation by hormones and growth factors depending on stage of differentiation [Aronow et al., 1990; Shalhoub et al., 1992; Breen et al., 1994], suggesting that these differentiation factors may be useful for the further characterization of the differentiation process of HOS 58 osteosarcoma cells.

MATERIALS AND METHODS

Cell Culture

All cell culture media and fetal calf serum (FCS) were purchased from Biochrom (Berlin, FRG). Cell culture disposables were purchased from Nunc (Wiesbaden-Biebrich, FRG) or Greiner (Solingen, FRG) and medium supplements (antibiotics, glutamine) from GIBCO/BRL (Eggenstein, FRG). Standard laboratory reagents were purchased from Sigma (Deisenhofen, FRG), if not noted otherwise.

HOS 58

The human osteosarcoma cell line HOS 58 was derived from an osteosarcoma of the leg of a 21-year-old man. Originally, the osteosarcoma tissue was removed and transplanted into nude mice [Groscurth et al., 1982], pieces of heterotransplants were repassaged and osteosarcoma cells for suspension cultures subsequently isolated. A stable cell line was established and characterized, demonstrating a doubling time of 36 h, cAMP increase after application of PTH and osteocalcin production after stimulation by calcitriol [Kern et al., 1990; Schulz et al., 1993]. HOS 58 cells express a rather differentiated phenotype with further maturation but lack mineralization during a culture period of 21 days [Siggelkow et al., 1998].

Human Bone-Derived Cell Culture

Primary human bone cell cultures were prepared from bone specimen as previously described [Siggelkow et al., 1999a] based on the original method by Beresford [Beresford et al., 1984; Auf'm Kolk et al., 1985]. Bone cells were grown out of explants, maintained in medium (DMEM with 10% fetal calf serum (FCS), glutamine (58.5 μg/ml), penicillin (100 U/ml), and streptomycin (100 μg/ml)) at 37°C in a humidified 95% air/5% CO_2 atmosphere. Cultures were initiated within 3 h of receiving the bone chips and fed twice a week.

Stimulation of Cultures

HOS 58 cells from passage number 72 were plated to reach confluence at day 7 (1.3×10^4 cells/cm^2) in 75-cm^2 flasks (for Northern blot), 6-well plates (for protein analysis, RT-PCR), or 24-well plates (electron microscopy) with ISCOVE medium supplemented with 10% FCS, 1% glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin. Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C. Medium was changed three times a week and 24 h before any analysis. Gene expression by Northern blot analysis and protein secretion/activity of AP, Oc, and Col 1 was investigated at day 7, 14, 21, and 28. At each timepoint, the monolayer was washed twice

with fresh DMEM medium containing 1% BSA (Sigma), $MgSO_4$ 0.02% and penicillin (100 U/ml), and streptomycin (100 μg/ml)) to remove FCS, followed by incubation with 10^{-9} M 1, 25-$(OH)_2$-D_3 (D3, Roche, Grenzach-Wyhlen, Germany) or solvent (ethanol < 0.01%) for 48 h at 37°C. In addition, cells were analyzed at identical timepoints by treatment with human transforming growth factor beta 1 (TGF-beta, 0.1 ng/ml, PromoCell, Heidelberg) or Dexamethasone (Dex,10^{-8} M, Sigma, München) for 48 h for gene expression by RT-PCR and protein synthesis. Untreated control cultures were prepared using the same manipulatons.

Mineralization was determined by energy-dispersive X-ray microanalysis (EDX-analysis) on day 28, after stimulation with beta-glycerophosphate (bGP, 2 mM), ascorbic acid (50 μg/ml) (added after confluence (day 7) and daily thereafter), and after addition of TGF-beta (0.1 ng/ml), Dex (10^{-8} M), and 10^{-9} M D3 in different combinations.

Cellularity Assay

Cell numbers were determined by quantifying total DNA content. Cellular DNA was measured in cell lysates using the Hoechst fluorescent dye [Hoechst 33258; Rago et al., 1990]. Fluorescence was quantified with a Cytofluor 2300 plate reader (Millipore, Eschborn, Germany). Cell numbers were determined by hemocytometer counting.

Analysis of Alkaline Phosphatase, Procollagen I, and Osteocalcin

AP activity was assayed in cell lysates by determining the release of *p*-nitrophenol from *p*-nitrophenyl phosphate at 37°C and pH of 10.5 as previously described [Siggelkow et al., 1999a]. Samples were measured in duplicate, and activity reported as nanomole per microgram DNA per minute incubation time [modified after Boyan et al., 1989]. The secreted carboxyterminal peptide of procollagen I (CICP) was measured by ELISA (Metra Biosystems Palo Alto, CA) in culture supernatants harvested 24 h after feeding. Collagen type I content was calculated from the c-terminal propeptide concentration [Melkko et al., 1990; Jukkola et al., 1991] measured in duplicate samples. Values were adjusted for the background levels of CICP present in the culture medium. The detection range of the kit was 1–80 ng/ml (intra-assay precision: 5.5–6.8%, interassay precision: 5.0–7.2%). Determination of Oc was performed in duplicate with an immunoradiometric assay (Nichols, Bad Nauheim, Germany); the detection range of the kit was 0.9–267 ng/ml (intra-assay precision: 8–10%, interassay precision: 4.8–9.8%). Values were corrected for interfering substances present in the culture medium.

RNA Isolation and Northern Blot Analysis

Total RNA was prepared using the RNeasy total RNA extraction kit from Qiagen (Hilden, Germany). Northern blot analysis was done as previously described [Siggelkow et al., 1999a]. 32P-labeled cDNA probes were synthesized by random priming [Feinberg and Vogelstein, 1983; Stratagene, Heidelberg]. Following hybridization, blots were washed under stringent conditions. Autoradiographs were quantified by laser densitometry (Biometra, Göttingen, Germany) and calculated in arbitrary OD units with the maximum absorbance on each blot set at 100 U. Ethidium bromide staining of 28 S ribosomal RNA was documented in gels before and in nylonfilters after blotting to control for equal loading of each transcript. Values represent the mean of three different cultures, unless stated otherwise.

cDNA Probes

Col 1 expression was measured using the rat proalpha(I) collagen cDNA clone pHCAL1U [Vuorio et al., 1987] detecting both 5.9 and 7.2 kb transcripts. The AP human cDNA probe was the 2.5-kb insert of pAT153 [Weiss et al., 1986] recognizing a transcript of 2.6 kb. Oc expression was analyzed using the human 1.2 kb-Sac I fragment of SP 65 [Celeste et al., 1986] with a transcript size of 0.6 kb.

RT-PCR

Total RNA was prepared using the RNeasy total RNA extraction kit from Qiagen (Hilden, Germany). cDNA was synthesized from 1 μg of total RNA in a 40 μl reaction mixture containing 3 mM $MgCl_2$, 75 mM KCl, 50 mM Tris-HCl (pH 8.3), 10 mM DTT, dCTP, dGTP, dATP, and dTTP each at 0.4 mM (Roche, Mannheim, Germany), 40 U of RNase inhibitor (Roche, Mannheim, Germany), 400 U of M-MLV reverse transcriptase (Gibco BRL, Karlsruhe, Germany), and 80 pmol of poly-dT15 primer (Roche, Mannheim, Germany). Reactions were incubated for 1 h at 38°C and 10 min at 72°C.

Aliquots of the cDNA were subsequently amplified by PCR using a 25 μl reaction mixture containing 20 pmol of 5′ and 3′ primer each, 23 μl Platinum® PCR SuperMIX (55 mM KCl, 22 mM Tris-HCl (pH 8.4), 1,65 nM $MgCl_2$ and dCTP, dGTP, dATP, and dTTP each at 0.22 mM and 1 U of Taq polymerase with Platinum Taq antibody (Gibco BRL, Karlsruhe, Germany). Each cDNA sample was amplified in duplicate.

Semiquantification of RT-PCR products was done using a competitive PCR approach by including exogenous DNA competitors ("mimics") as internal control [Viereck et al., 2002]. Mimic sequences were selected that differed in size from the target amplification product and which competed with the target cDNA for the primers, were synthesized using the mimic construction kit (PCR mimic construction kit, Clontech, Palo Alto). To quantify the amount of target cDNA serial dilutions of the internal standard were added to the cDNA amplification reactions. The amount of target cDNA present in the samples was estimated by comparing target and internal standard amplification products using an ethidium bromide staining of agarose-gel separated PCR reaction products. RT/PCR was used to analyze mRNA expression of AP, Col 1, Oc, L7, a "housekeeping" ribosomal gene (primer sequences as published by Rickard et al. [1996]) and core binding factor alpha 1, an osteoblast specific transcription factor (Cbfa1, sense: 5′-CCACCTCTGACTTCTGCCTC-3′; antisense: 5′-GACTGGCGGGGTGTAAGTAA-3′). Gene expression was calculated as the ratio of signal intensities of target to internal standard and were normalized to L7 gene expression. PCR was performed in a Primus PCR-Thermocycler (MWG-Biotech, Ebersberg, Germany). PCR reactions were carried out in 15 μl reactions at a cycle number ensuring a linear amplification profile (AP: 2 min at 94°C, 28 cycles (of 30 sec at 94°C, 45 sec at 55°C, 90 sec at 72°C), 7 min at 72°C; Oc: 2 min at 94°C, 33 cycles (of 30 sec at 94°C, 45 sec at 55°C, 90 sec at 72°C), 7 min at 72°C; Col 1: 2 min at 94°C, 33 cycles (of 30 sec at 94°C, 45 sec at 55°C, 90 sec at 72°C), 7 min at 72°C; Cbfa1: 2 min at 95°C, 30 cycles (of 30 sec at 95°C, 30 sec at 59°C, 1 min at 72°C), 10 min at 72°C; L7: 2 min at 94°C, 22 cycles (of 60 sec at 94°C, 1 min at 54°C, 2 min at 72°C), 10 min at 72°C; Oligonucleotides were synthesized by MWG-Biotech (Ebersberg, Germany). Reaction products were analyzed by electrophoresis of 15 μl samples in 1.5% (w/v) agarose gels, and visualized by ethidium bromide staining under UV light. A 100-bp DNA ladder (Gibco BRL, Karlsruhe, Germany) served as size marker. To ensure specifity of the PCR products, sequence analysis of the amplification product was performed using the Abi Prism system from Perkin Elmer (Weiterstadt, Germany). Gels were recorded by a Kodak Digital Science Kds 1d camera system and band intensities were quantified by an Electrophoresis Documentation and Analysis System 120 (Kodak, Stuttgart, Germany).

Scanning Electron Microscopy and Energy-Dispersive X-ray Microanalysis (EDX)

Samples were grown in 24-well culture plates and fixed with a fixation solution containing 3% glutaraldehyde (w/v) and 5% formaldehyde (w/v) in phosphate buffer for 1 h on ice. After several washing steps with phosphate buffer, samples were cut out of culture plates and dehydrated with a graded series of ethanol (10, 30, 50, 70, 90, 100%) and critical-point dried with CO_2 (Balzers CPD030, Liechtenstein). The dried confluent cells were then partially removed by a strong air-stream passed through a small pipette tip. This procedure allows for detection of possible mineralization processes underneath the confluent cell layer on the growth support or in collagen containing matrix regions. Subsequentely, the samples were sputter-coated with a gold film of approximately 10 nm in thickness (Bal-Tec SCD040, Liechtenstein). Samples were examined in a Zeiss field emission scanning electron microscope DSM982 Gemini (Zeiss, Oberkochen). Energy dispersive X-ray analysis (EDX) was carried out with an Link-ISIS 300 system (Si(Li)-detector, Oxford Instruments, Wiesbaden, Germany) [Akesson et al., 1994]. Samples were examined in the DSM982 spot-mode with an acceleration voltage of 15 kV, a working distance of 8 mm, an aperture of 60 μm, and using a 2 min collection time for acquisition of spectra data. All spectra were scaled to 15 counts/sec. One has to be aware that the detected calcium phosphate crystals do not automatically reflect proper mineralization of a collagenous matrix which would have to be proven independently.

Statistical Analysis

All values are expressed as mean ± SEM (standard error of the mean). Comparison

between groups was made by using the Wilcoxon non-parametric test unless otherwise noted. For correlation between cell number and DNA data Spearman's Rank correlation was applied. Significant differences ($P < 0.05$, $P < 0.01$, and $P < 0.001$) are marked with *, **, and ***, respectively.

RESULTS

Proliferation of HOS 58 Osteosarcoma Cells

The adherent cell number increased steadily during the initial 21 days, plateauing thereafter (Fig. 1) as already shown previously [Siggelkow et al., 1998]. The preconfluent doubling time of HOS 58 cells was 27 h (confluence was reached by day 7). Postconfluent cells exhibited a doubling time of 80 h. This slower growth was not caused by an increased number of nonadherent cells but was due to a flattening of the exponential growth curve (data not shown). Values for DNA content ($r = 0.99$, $P < 0.05$) and total protein ($r = 0.98$, $P < 0.05$) both correlated strongly with total cell numbers in both control and treated cultures. We have therefore systematically used DNA values for the correction of AP, Oc, and Col I protein data.

D3 significantly stimulated cell numbers at all timepoints investigated, with the maximal effect occurring on day 14 (1.8-fold) in postproliferative cultures compared to day 7, 21, and 28 (1.4-fold) (Fig. 1). Proliferation in HOS 58 cells was significantly stimulated at all timepoints by the 48 h application of D3. There was no influence of TGF-beta on cell proliferation at any timepoint (data not shown). We saw a small but significant stimulatory effect of Dex on proliferation at day 28 (data not shown).

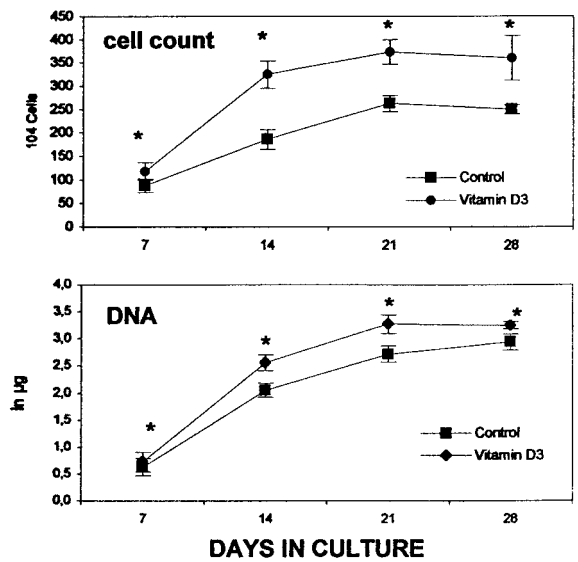

Fig. 1. Proliferation as measured by cell count and DNA assay in HOS 58 osteosarcoma cells at four different timepoints: day 7 (confluence), day 14, day 21, and day 28 after 48 h incubation with 10^{-9} M 1,25-$(OH)_2$-D_3 (Vitamin D3) or solvent (control). Data presented as mean ± SEM (n = 4), signifcant differences as calculated by Wilcoxon non-parametric test, $*P < 0.05$.

Alkaline Phosphatase Gene Expression and Activity

While mRNA levels of AP were constant from day 7–28, the AP enzymatic activity increased by 1.9-fold, plateauing by day 21 ($P < 0.05$) (Fig. 2A,C). In the developmental sequence described by Owen et al. [1990], AP activity increased from low levels during the phase of rapid cell proliferation and reached maximum expression during the phase of matrix synthesis, typically decreasing sharply at the onset of mineralization [Owen et al., 1990]. In our experiments, we observed differences between the AP activity and expression in 75-cm^2 culture flasks (Fig. 2A,C) and 6-well plates (Fig. 2B,D). This could be due to differences in flask size resulting in an altered differentiation sequence. Therefore, we analyzed AP activity and expression in HOS 58 cells under mineralizing conditions by adding beta-glycerophophate and ascorbic acid over the 4-week culture. Under these conditions, we were able to reproduce the developmental sequence described by Owen et al. [1990]; data not shown.

We demonstrated an inhibiting effect of D3 on AP mRNA and protein levels in HOS 58, an effect still present for mRNA even at day 28 in culture (Fig. 2A,C).

TGF-beta has been suggested to induce matrix maturation by decreasing AP gene expression [Lomri and Marie, 1990]. In HOS 58 cells, AP gene expression (Fig. 2D) and activity (Fig. 2B) were both decreased by TGF-beta in the late postconfluent stages. Gene expression started to decrease from day 14, falling to 66% of control levels by day 28 (Fig. 2D).

Dex significantly decreased AP activity from day 14 with no effect thereafter (Fig. 2B). There was no influence of Dex on AP gene expression at confluence. At the beginning at day 14, AP mRNA was decreased to 62% of basal values until late in culture (Fig. 2D).

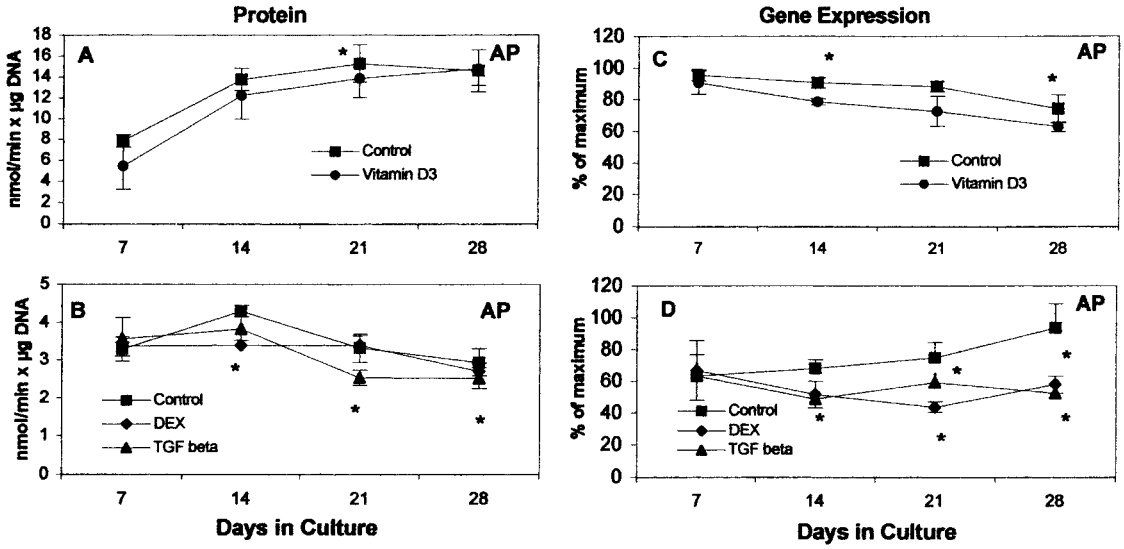

Fig. 2. Alkaline Phosphatase (AP) activity and gene expression during in vitro culture of HOS 58 osteosarcoma cells. Cellular RNA from four independent experiments was isolated at the days indicated and assayed by Northern blot analysis (**C**) or RT-PCR (**D**). On the right, the relative gene expression in percentage of maximum expression (mean ± SEM) under the influence of stimulating agents or control is demonstrated (C–D). Expression was quantified by laser densitometry, results were plotted against the maximum expression of each transcript. On the left side, upper panel (**A**) AP activity under the influence of 10^{-9} M 1,25-$(OH)_2$-D_3 (Vitamin D3) or solvent (control). On the left side lower panel (**B**) AP activity under the influence of TGF-beta (0.1 ng/ml), Dexamethasone (Dex,10^{-8} M) or solvent (control). Data presented as mean ± SEM (A: n = 4 or B: n = 6), signifcant differences as calculated by Wilcoxon non-parametric test, $^*P < 0.05$.

Procollagen I

There were only minor changes in collagen gene expression and protein secretion in untreated HOS 58 cells from day 7–28 in culture (Fig. 3A,C). CICP secretion increased 1.5-fold from day 7–14 ($P < 0.05$) and significantly decreased thereafter until day 28 (Fig. 3A, $P < 0.05$).

A small but significant inhibitory effect of D3 on collagen gene expression was detectable at confluence. At that timepoint, CICP secretion was significantly stimulated (Fig. 3A,C). In HOS 58 cells, TGF-beta showed only a non-significant stimulating effect on CICP at confluence (day 7) (Fig. 3B). In contrast to the effect on CICP, TGF-beta decreased Col I gene expression after confluence (Fig. 3D).

Dex inhibited the Col I gene expression at all timepoints investigated, and only at day 21 the difference between control and Dex stimulated cultures was not significant (Fig. 3D). No significant influence of Dex on CICP was detectable at any timepoint investigated (Fig. 3B).

Osteocalcin

Basal Oc gene expression was detectable only after prolonged exposure of hybridizations due to overall low expression (Fig. 4). From day 7 to 28, basal Oc gene expression increased significantly by 10.4-fold (Fig. 5C,D). In contrast to these changes in gene expression, the basal values for Oc protein were already maximal by day 7 and decreased significantly by 3.3-fold at day 14 and stayed unchanged thereafter (Fig. 5A).

D3 stimulated Oc expression showed 100-fold increased levels at day 7 compared to control. At day 28, a 37-fold stimulation of Oc gene expression was still inducible (Figs. 4 and 5C). Because a precise quantification of basal and stimulated gene expression was not feasable due to over and underexpression on X-ray films, we repeated these experiments and analyzed them by RT-PCR. The D3 stimulated cultures showed a 21-fold increase in Oc gene expression at day 7, decreasing to 2-fold stimulation at day 14–28 (not shown). Oc protein secretion was maximally stimulated by D3 at day 7 at its peak levels (7.1-fold, $P < 0.05$) and from day 14 to 28 decreased to only 2-fold. D3 stimulated Oc protein decreased significantly from day 7 compared to day 14 (11.2-fold, Fig. 5A). The differences in Oc protein measurements at the various timepoints could either be due to changes in Oc synthesis or secretion into the medium. We therefore measured Oc in the cell

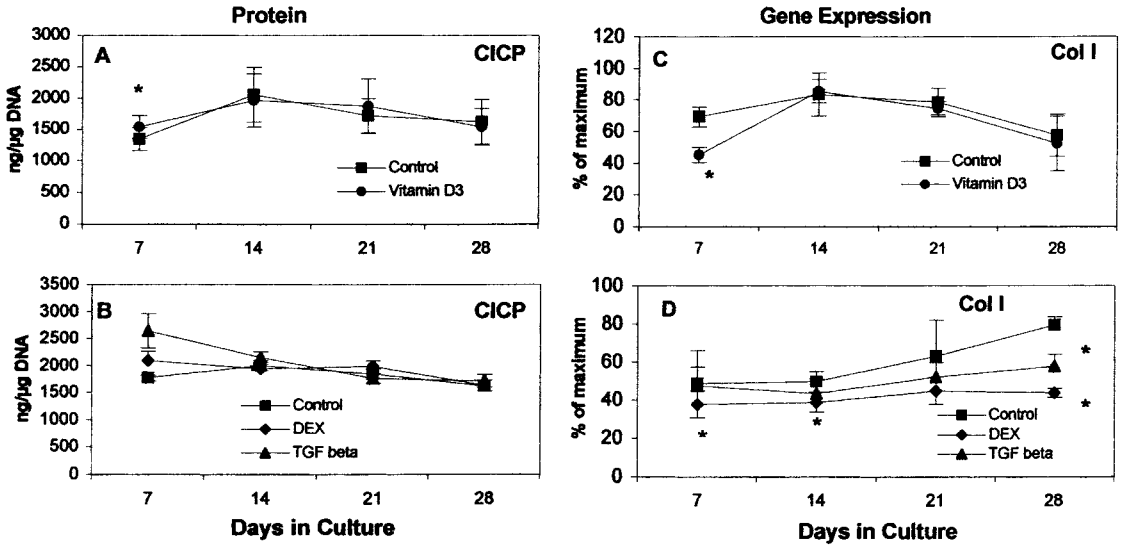

Fig. 3. Collagen protein secretion (CICP) and gene expression during in vitro culture of HOS 58 osteosarcoma cells. Cellular RNA from four independent experiments was isolated at the days indicated and assayed by Northern blot analysis (**C**) or RT-PCR (**D**). On the right, the relative gene expression in percentage of maximum expression (mean ± SEM) under the influence of stimulating agents or control is demonstrated (C–D). Expression was quantified by laser densitometry, results were plotted against the maximum expression of each transcript. On the left side, upper panel (**A**) CICP under the influence of 10^{-9} M 1,25-$(OH)_2$-D_3 (Vitamin D3) or solvent (control.) On the left side lower panel (**B**) CICP under the influence of TGF-beta (0.1 ng/ml), Dexamethasone (Dex,10^{-8} M) or solvent (control). Data presented as mean ± SEM (A: n = 4 or B: n = 6), signifcant differences as calculated by Wilcoxon non-parametric test, $^*P < 0.05$.

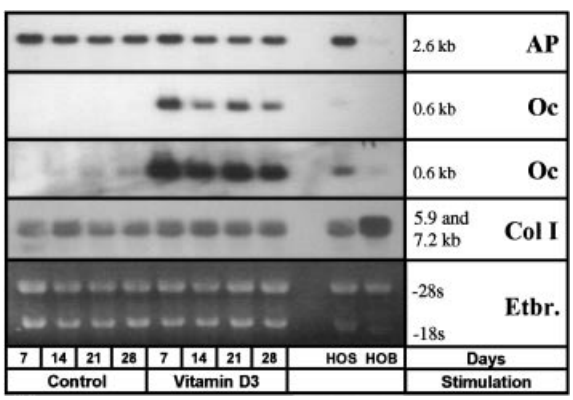

Fig. 4. Gene expression during in vitro culture of HOS 58 human osteosarcoma cells. Cellular RNA from four independent experiments was isolated under the influence of 10^{-9} M 1,25-$(OH)_2$-D_3 (Vitamin D3) or solvent (control) at the days indicated (7, 14, 21, and 28 days) and assayed by Northern blot analysis. HOS: HOS 58 pool mRNA to compare different northern blots, HOB: pHOB pool mRNA to compare to human osteoblasts. Representative northern blots are depicted to illustrate alterations in mRNA expression. The osteoblast-characteristic gene alkaline phosphatase (AP, 2.6 kb) is shown in the upper panel. Osteocalcin (Oc) is depicted after 4 h and 7 days exposition of X-rays to demonstrate the low but increasing expression in control cultures from day 7 to day 28. As matrix protein procollagen I (Col I, 5.9 and 7.2 kb) expression is demonstrated. The ethidium bromide staining (Etbr), which shows equal gel loading, was used as reference.

pellet and detected a 2.2–2.8 ther increased Oc synthesis at the four different timepoints (data not shown). We conclude that the high increase in Oc in culture medium at confluence (day 7) is caused by an increase in Oc biosynthesis at day 7 rather than by a decreased secretion at other stages of the culture.

TGF-beta did not influence Oc protein at day 7. A significant stimulating effect was detectable at day 21 but the overall influence on Oc secretion was discrete (Fig. 5B). TGF-beta stimulated Oc mRNA being significant from day 14 to 21 when basal levels were low (Fig. 5D). Gene expression was decreased by 47% at the latest stage and highest level (day 28, Fig. 5D).

Dex decreased Oc gene expression significantly from day 14 to 28, by about 80% (Fig. 5D). Oc protein secretion was inhibited by Dex, but only significantly at day 14 by 40% (Fig. 5B). In our opinion, this indicates that an effect of Dex is only present at higher levels of mRNA and protein secretion.

Scanning Electron Microscopy and Energy-Dispersive X-ray Microanalysis (EDX)

Figure 6 depicts electronmicropgraphs and EDX analysis scans of HOS 58 cells treated

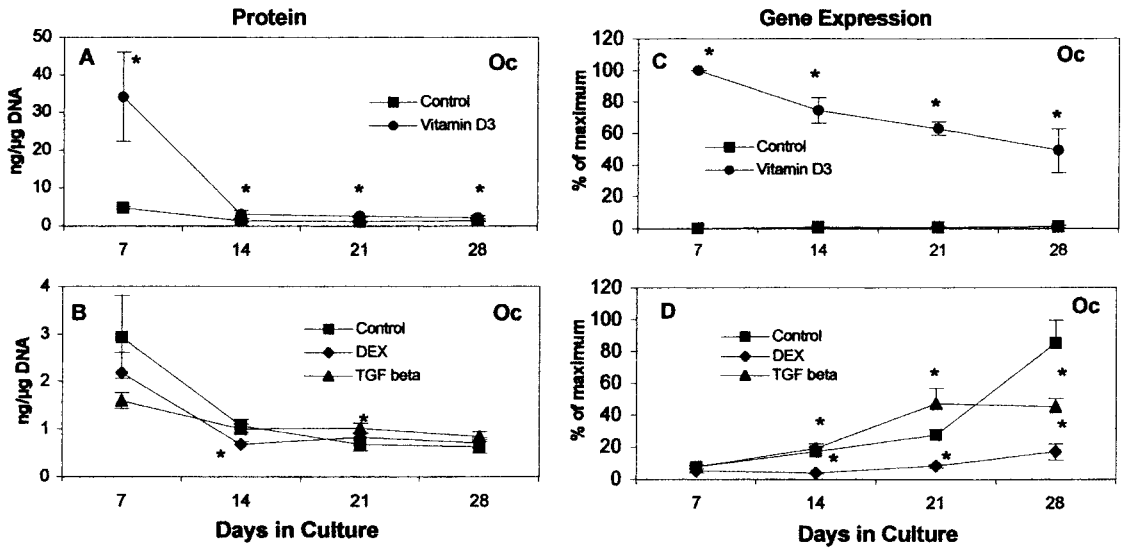

Fig. 5. Osteocalcin (Oc) protein secretion and gene expression during in vitro culture of HOS 58 osteosarcoma cells. Cellular RNA from four independent experiments was isolated at the days indicated and assayed by Northern blot analysis (**C**) or RT-PCR (**D**). On the right, the relative gene expression in percentage of maximum expression (mean ± SEM) under the influence of stimulating agents or control is demonstrated (C–D). Expression was quantified by laser densitometry, results were plotted against the maximum expression of each transcript. On the left side, upper panel (**A**) Oc under the influence of 10^{-9} M 1,25-$(OH)_2$-D_3 (Vitamin D3) or solvent (control). On the left side lower panel (**B**) Oc under the influence of TGF-beta (0.1 ng/ml), Dexamethasone (Dex,10^{-8} M) or solvent (control). Data presented as mean ± SEM (A: n = 4 or B: n = 6), significant differences as calculated by Wilcoxon non-parametric test, $^*P < 0.05$.

with ascorbic acid, bGP, TGF-beta, and Dex for 21 or 28 days. HOS 58 cells appear irregular in shape and exhibit different sphere-like particles on the cell surface. No deposition of material on the culture plate surface is visible. EDX-analysis of these particular structures revealed no calcium signal and only a very weak oxygen signal. Identical results were observed after HOS 58 cells were treated with D3, TGF-beta/D3 or Dex/D3 in addition to ascorbic acid and bGP (data not shown). The EDX-analysis results demonstrate that HOS 58 cells exhibit no mineralization process. Figure 7 depicts the mineralization process detected in control cultures of pHOB. Here EDX-analysis was able to demonstrtate the presence of calcium, oxygen, and phosphorus signals, which is strong evidence for mineralization of these cells.

Expression of Core Binding Factor Alpha 1 (Cbfa1)

To investigate if the lack of mineralization was due to low or missing Cbfa1 levels, we quantified Cbfa1 mRNA expression in HOS 58 cells maintained in culture over 32 days. Cbfa1 gene expression increased from day 1 to 4 and up to 16-fold levels at day 20 with a decrease thereafter (Fig. 8). This demonstrates that the defect in mineralization could not be explained by lack of Cbfa1 gene expression.

DISCUSSION

HOS 58 osteosarcoma cells investigated during prolonged in vitro culture showed many typical characteristics of normal differentiating osteoblastic cells. In this study, we have attempted to answer two questions: (1) to what extent are characteristic osteoblast proteins physiologically regulated in the HOS 58 cell line and (2) can functional derangements be identified? We give a comprehensive overview of our HOS data in Table I. In addition, for better understanding of the detailed discussion, we show a summary of the published data on primary osteoblast differentiation systems in rat and human in Table II. Further data on other osteoblast systems are mentioned in the text.

The low initial doubling time (20 h) is typical for the transformed nature of osteosarcoma cells [Rodan et al., 1987; Fournier and Price, 1991; Clover and Gowen, 1994] compared to primary osteoblastic cells which double every 120–125 h [Auf'm Kolk et al., 1985; Clover and Gowen, 1994]. At high levels of confluence, HOS 58 cells

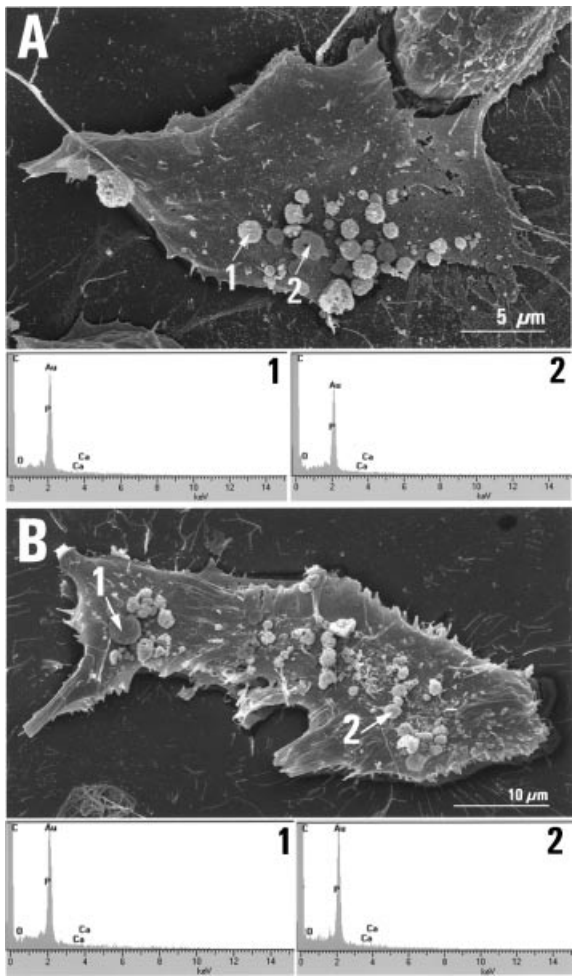

Fig. 6. EDX analysis of HOS 58 cells treated with TGFβ/Dex for 21 days (**A**) or 28 days (**B**) HOS 58 cells exhibit sphere-like particles on the cell surface. EDX analysis of two of these particles for each day (arrows) revealed no calcium signal and a very weak oxygen signal most properly resulting from the culture plate. The gold signal represents the gold film covering the samples for conductivity.

showed a slowing down of cell doubling, presumably due to contact inhibition.

1,25 Dihydroxy-Vitamin D3 is a potent differentiation factor for osteoblasts, inducing the transition from proliferation to differentiation [Owen et al., 1991; Bonewald et al., 1992], accompanied by a decrease in cell doubling [Chen et al., 1983] while stimulating expression of markers of DNA-synthesis in more differentiated cells [Owen et al., 1991]. In pHOB, the effect of D3 seemed to be stage dependent because it stimulated proliferation in more confluent cells [Siggelkow et al., 1999b]. Therefore, the proliferation stimulating effect of D3 on HOS 58, an already highly differentiated cell, is

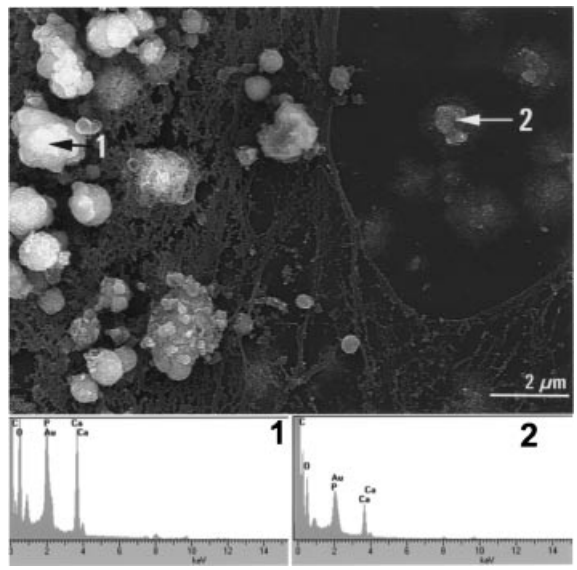

Fig. 7. EDX analysis of pHOB cells as control. EDX spectra show strong signals of calcium, oxygen, and phosphorus demonstrating the identical analysis settings for analysis of HOS 58 cells.

in accordance with this biphasic effect of D3 seen in rat calvarial osteoblasts [Owen et al., 1991].

No effect of TGF-beta or Dex on HOS 58 proliferation was detectable. TGF-beta has two major biological effects on bone cells: growth inhibition and the stimulation of extracellular matrix formation [Bonewald, 1999]. A TGF-beta mediated inhibition of proliferation has been shown for several, mostly non-human, osteosarcoma cells [Noda and G.A, 1987; Pfeilschifter et al., 1987; Bonewald et al., 1992; Centrella et al., 1995], as well as for primary rat osteoblast systems [Guenther et al., 1988; Hock et al.,

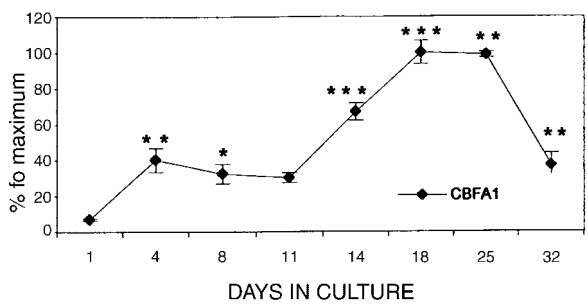

Fig. 8. Gene expression of Cbfa1 in HOS 58 cells during 32 days in culture. Mean ± SEM of five different experiments analyzed by RT-PCR as described in Materials and Methods. Values were normalized to the house keeping gene L7. To statistically compare the timepoints, Tukey's multiple comparison test was used. Stars represent the difference to the preceding timepoint with $***P < 0.001$, $**P < 0.01$, and $*P < 0.05$.

TABLE I. Summary of the Data in HOS 58 Cells

	Confluence				Post confluent				Late post confluent			
Day	7				14				21 and 28			
Stimulation	Basal	D3	TGF-β	DEX	Basal	D3	TGF-β	DEX	Basal	D3	TGF-β	DEX
Proliferation	Confluence at day 7	↑	—	—	↓	↑	—	—	—	↑	—	↓ —
AP mRNA	Medium	— ↓	—	ns ↓	—	↓	—	→	→	→	→	→
AP activity	Medium	—	—	—	↑↑	—	→	→	Max ↑↑	— ↓	→	ns↓
Oc mRNA	Low	× 800↑↑	—	—	↑	× 36 ↑↑	↑	→	↑	× 36↑↑	→	→
Oc protein	Max	× 7.2↑↑	—	—	→	× 2↑	—	—	→	× 2↑	—	→

Arrows from stimulated cultures compared to control, arrows of basal values compared to the timepoint before, –: no significant change, grey arrows and ns: tendancy but data not significant.
AP, alkaline phosphatase; Oc, osteocalcin, D3: 10^{-9} M 1,25-$(OH)_2$-D_3; TGF-β, human transforming growth factor beta 1 (0.1 ng/ml); DEX, dexamethasone (10^{-8} M) for 48 h at 37°C.

1990]. In contrast, proliferation stimulating effects of TGF-beta have been reported in bone marrow stromal cells (putative osteoblast precursors) and in pHOB cells [Ingram et al., 1994; Liu et al., 1999; Kassem et al., 2000]. In a differentiation model of rat calvarial cells, a biphasic effect of TGF-beta has been shown, with a negative effect on growth before and positive effect on growth after confluency. This phenomenon may explain the contradictory results obtained in other models demonstrating a differentiation-dependent difference in the effect of TGF-beta. In osteoblast cell systems, the long term application of Dex is frequently used to promote the differentiated phenotype [Wong et al., 1990] while short-term application has variable effects [Shalhoub et al., 1992]. We used a short-term application of Dex and compare our observations only with those using similar treatment regimens. A growth inhibiting effect of Dex was reported in rodent osteoblasts [Chen et al., 1983, 1991; Lukert et al., 1991], in pHOB cells [Kasperk et al., 1995a] and in human osteosarcoma cells [Song, 1994]. The lack of an inhibiton of cell doubling by Dex or TGF-beta in our study of HOS 58 cells is, in our opinion, a consequence of the transformed tumor cell phenotype.

The AP glycoprotein is localized in matrix vesicles and the plasma membrane, and involved in matrix formation and calcification [Bonewald et al., 1992], although the exact function of AP in this process is still unknown. HOS 58 cells expressed high basal AP mRNA and enzyme activity at the beginning of culture, which further increases in parallel to the downregulation of proliferation, as described for primary cells [Owen et al., 1990; Stein et al., 1990]. Although in these experiments, the AP gene expression did not show exactly the same sequence as rat calvarial cells and pHOB [Owen et al., 1990; Siggelkow et al., 1999a], this is due to missing bGP. The effect of D3 on AP in the various osteoblast model systems remains controversial. D3 has been shown to both positively and negatively regulate the gene expression of osteoblast phenotypic markers, depending on both the duration of hormone treatment and initial basal levels of gene expression [Owen et al., 1991]. In the rat calvarial osteoblast model, a decreasing effect of D3 on AP gene expression and protein activity until day 18 (maturation stage) in culture has been demonstrated, whereas after day 24 both mRNA and

TABLE II. Summary of Published Data on Rat or Human Differentiation Models in Primary Osteoblastic Cells Based on Time in Culture or Confluence

		Preconfluence/confluence								Post confluent								Late post confluent							
Day		7								14								21 and 28							
Stimulation		Basal	Ref.	D3	Ref.	TGF-β	Ref.	DEX	Ref.	Basal	Ref.	D3	Ref.	TGF-β	Ref.	DEX	Ref.	Basal	Ref.	D3	Ref.	TGF-β	Ref.	DEX	Ref.
Parameter	System																								
Proliferation	Human	Confluence at day 10	1	↓	4	↑	4	nd		↓	8	↑	6	nd		↓	5	↓	8	nd		nd		nd	
	Rat	Confluence at day 7	6	↓	2	↓	1	nd		↓	2	↑	6	↑	1	↓↑	6,7	↓	6	nd		↑	1	↑—	7
AP mRNA	Human	Low	8	—	9	nd		nd		Max↑↑	9	—	9	nd		nd		↓	8			nd		nd	
	Rat	Low	6	↓	6	—	1	nd		Max↑↑	6	↓	6	↓	1	↓	7	↓	6	↑	6	↓	1	↑	7
AP activity	Human	Low	8	↑	9	↑	4	↑	5	Max ↑↑	8	—	9	nd		nd		↓	8	—	9	nd		nd	
	Rat	Low	6	↓	6	—	1	nd		Max ↑↑	6	↓	6	nd		nd		↓	6	↑	6	↓	1	nd	
Oc mRNA	Human	Low	8	×60↑↑	9	nd		nd		↑	9	↑	9	nd		nd		—	8	nd		nd		nd	
	Rat	Low	6	↑↑	6	nd	1	nd		↑	6	↑	6	nd	1	—	7	↑↑	6	↑	6	nd		↑	7
Oc protein	Human	—	8	×13↑↑	9	—	4	↓	5	↑	9	↑	9	nd		nd		—	8	nd		nd		nd	
	Rat	—	6	↑↑	6	—	1	nd		↑	6	↑	6	—	1	nd		↑↑	6	↑	6	↓	1	↑	7

Arrows from stimulated cultures compared to control. Arrows of basal values compared to the timepoint before. —, no significant change; Ref., references; nd, no available data in a differentiation system.

1: Breen et al., 1994, 2: Chen et al., 1983, 3: Chen et al., 1991, 4: Ingram et al., 1994, 5: Kasperk et al., 1995a, 6: Owen et al., 1991, 7: Shalhoub et al., 1992, 8: Siggelkow et al., 1999a, 9: Siggelkow et al., 1999b.

AP, alkaline phosphatase; Oc, osteocalcin, D3: 10^{-9} M 1,25-$(OH)_2$-D_3; TGF-β, natural human transforming growth factor beta 1 (0.1 ng/ml); DEX, Dexamethasone (10^{-8} M) for 48 h at 37°C.

For this table only data from differentiation models were used. Data not related to time in culture or confluence are described in the text. Not all authors mentioned whether the data were significant.

Ref., references; nd, no available data in a differentiation system.

protein levels of AP were stimulated [Owen et al., 1991] in the presence of ascorbic acid and bGP. In contrast, in pHOB, D3 stimulated AP gene expression and activity during proliferation right up to the point of confluence in pHOB [Siggelkow et al., 1999b]. Contradictory results were also reported by other groups when D3 variously inhibited [Broess et al., 1995] or stimulated [Bonewald et al., 1992] AP. In MG-63 human osteosarcoma cells, AP is stimulated by D3 [Clover and Gowen, 1994] but there is no effect in either the OHS-4 or HOS-TE-85 human osteosarcoma cell lines [Fournier and Price, 1991; Clover and Gowen, 1994]. Questions remain whether the effect of D3 is dependent on ascorbic acid and bGP, and which phenotype should be regarded as being the most physiological. Therefore, we do not know whether the inhibitory effect of D3 on AP actvity and gene expression observed in HOS 58 cells is actually physiological. The inhibitory effects of TGF-beta and Dex on AP expression depended on the time of the culture and show that HOS 58 cells at different maturation stages are differentially regulated. In addition, the effect of both factors corresponds to data obtained in both primary rat and human osteoblast models [Shalhoub et al., 1992; Breen et al., 1994; Ingram et al., 1994; Liu et al., 1999; Kassem et al., 2000]. Therefore, in HOS 58 cells, AP seems to be expressed and regulated in a physiological manner.

Collagen gene expression and protein secretion were not differentially expressed in the HOS 58 system under the chosen conditions. A biphasic gene expression profile, with an early increase during proliferation followed by a decrease, has been show in other studies using rat [Owen et al., 1990, 1991] and chick [Gerstenfeld et al., 1988] calvaria-derived osteoblasts, and in bovine neonatal long bone osteoblasts [Ibaraki et al., 1992]. When HOS 58 cell cultures were previously analyzed up to day 7, maximal expression of Col1 was detected as early as day 4, subsequently decreasing [Siggelkow et al., 1998].

D3 had only minor effects on collagen in HOS 58 cells. Collagen mRNA and CICP levels were are also shown to be not influenced by D3 in a differentiation model of pHOB [Siggelkow et al., 1999b]. In mature rat calvarial osteoblasts, in contrast, a significant stimulatory effect on collagen gene expression was reported, albeit only when analyzing mineralized matrix in the presence of beta-glycerophosphate and ascorbic acid in addition to D3 [Owen et al., 1991].

In contrast to our results in HOS 58, TGF-beta was shown to stimulate matrix biosynthesis (procollagen I secretion) in rat calvarial cells, and other human and rodent systems [Hock et al., 1990; Strong et al., 1991; Bonewald et al., 1992; Breen et al., 1994; Ingram et al., 1994; Centrella et al., 1995; Kassem et al., 2000]. This would appear to be dependent on D3 and cell maturity, as is not detectable in the less differentiated bone marrow stromal cell system [Kassem et al., 2000]. The inhibitory effect of TGF-beta on collagen gene expression levels, we see in HOS 58, was identical to data reported using rat calvarial cells [Breen et al., 1994].

In contrast to the inhibitory effect of Dex in HOS 58 cells, no influence on procollagen I mRNA levels after 48 h stimulation with Dex was seen in rat calvaria cells [Shalhoub et al., 1992]. Whereas in HOS 58, there was no significant effect on CICP levels, CICP was strongly inhibited by Dex in pHOB [Kasperk et al., 1995a] when ascorbic acid was added to induce collagen secretion. Procollagen I expression and CICP levels have been shown to be dependent on ascorbic acid stimulation [Spindler et al., 1989; Owen et al., 1990; Franceschi et al., 1994; under these circumstances]. Most of the studies in differentiation models used ascorbate in additon to other factors [Chen et al., 1991; Lukert et al., 1991; Shalhoub et al., 1992; Breen et al., 1994; Delany et al., 1994; Lynch et al., 1995; Kasperk et al., 1995a; Lian et al., 1997]. To summarize our gene expression and protein data for collagen I, we conclude that in the absence of ascorbate there was no decisive influence of time in culture or of other applied factors on the differentiation in HOS 58 cells.

Oc, the predominant noncollagenous protein of bone, is a negative regulator of bone formation [Ducy et al., 1996]. In rat calvarial cells, it is expressed late in the developmental sequence, where its expression correlates with mineral deposition [Aronow et al., 1990; Lian and Stein, 1992]. In HOS 58 cells and pHOB basal levels of Oc gene expression are low early in culture [Siggelkow et al., 1999a,b] suggesting that expression at such an early stage is not per se a tumor cell trait. In contrast to the results in HOS 58 cells, which express maximal values of Oc protein at confluence, other osteoblast systems show an increase of Oc protein during all stages of differentiation and a parallel increase

of mRNA and protein levels [Owen et al., 1990; Siggelkow et al., 1999b]. The maximal effect of D3 on Oc gene expression and protein synthesis was seen at day 7 in culture. A similar stimulatory effect of D3 on Oc at an early developmental stages has been shown for rat calvarial cells [Owen et al., 1991] and pHOB [Siggelkow et al., 1999b]. Although the stimulatory effect of D3 on HOS 58 appears to be physiological, the magnitude of the increase is much higher than those reported in other systems.

The effect of TGF-beta on Oc in HOS 58 cells was stimulatory at lower levels and inhibitory at the highest level (significant only for gene expression), suggesting a differentiation-stage dependent effect. In rat calvarial osteoblasts, TGF-beta not only decreased Oc gene expression at late stages of culture but also inhibited nodule formation [Breen et al., 1994]. A negative effect of TGF-beta on Oc mRNA has also been described for both rat [Noda and G.A, 1987] and human [Pirskanen et al., 1994] osteosarcoma cells. A high Oc level seems to be a prerequisite because TGF-beta only inhibited the D3 stimulated Oc levels in different models of pHOB [Ingram et al., 1994]. In the absence of D3, no effect on Oc was seen in MG-63 cells [Bonewald et al., 1992; Liu et al., 1999; Kassem et al., 2000]. No stimulatory effect of TGF-beta on Oc similar to that we report using the HOS 58 cells has been described to date.

In HOS 58 cells, the effect of Dex seemed also to be differentiation dependent. In contrast to this observation, Oc could be stimulated by short-term application of Dex (48 h) in post-confluent fetal rat calvarial cells [Shalhoub et al., 1992], whilst in differentiation stages of rat 17/2.8 osteosarcoma cells, Dex showed no effect on Oc [Schepmoes et al., 1991]. This could be due to species differences as human [Subramaniam et al., 1992; Delany et al., 1994; Kasperk et al., 1995a] and rodent [Lian et al., 1997] osteoblast systems as well as human SAOS-2 osteosarcoma cells [Rao et al., 1996] all show an inhibition of Oc secretion by Dex.

In summary, we conclude that the decrease in Oc secretion, the level of D3 stimulated levels early in culture, as well as the effect of TGF-beta evident in HOS 58 cells cannot be explained by data of other authors, and that the effect of Dex on HOS 58 cells appears to be species dependent.

The data for Oc are also remarkable in a different regard in HOS 58 cells. The basal gene expression increased during culture, as shown for primary rat cells [Owen et al., 1990] and human osteoblasts [Siggelkow et al., 1999b]. However, in contrast to the latter models where protein secretion parallels gene expression, in HOS 58 cells basal Oc protein biosynthesis was maximal at an early stage and decreased thereafter. In other words, we can show a dissociation of Oc gene expression and Oc biosynthesis. Furthermore, Oc mRNA and biosynthesis in HOS cells were both stimulated by D3, as expected. Although in primary cell models, the maximal stimulation by D3 was seen at confluence [Owen et al., 1991; Siggelkow et al., 1999b], the increase in HOS 58 was much higher than in pHOB or rat calvarial cells. In addition, in HOS 58, during prolonged time in culture the stimulatory effect on Oc protein secretion was lower than in the other two models. Increase of Oc mRNA levels is normally paralleled by increased levels of transcription and protein biosynthesis [Owen et al., 1991; Lian and Stein, 1992]. This is obviously not the case in HOS 58 cells. We suggest from our data that this cell line might exhibit a translational defect in basal Oc biosynthesis, which results in a decrease in protein secretion despite the increase in gene expression.

We demonstrated in our study that, compared to pHOB, the HOS 58 cells show no mineralization capacity even when cultured with ascorbate, beta-glycerophosphate, D3, Dex, and TGF-beta in various combinations. In rat calvarial osteoblasts, nodule formation was visible after 35 days in culture in the presence of ascorbic acid and bGP alone. We were not able to induce mineralization even after 3 months of culture [Siggelkow et al., 1998]. The previously described inhibiting effect of TGF-beta on mineralization [Kato et al., 1988; Bonewald et al., 1992] could not be the reason for defective mineralization because we used identical conditions in the pHOB control. The AP regulation was not altered and could not explain this defect. In several in vitro systems, an increase in Oc expression has been shown to parallel the onset of mineralization [Stein et al., 1990; Owen et al., 1991] and therefore it was assumed for years that Oc was a prerequisite for mineralization. However, it has been recently demonstrated that Oc-deficient mice show no mineralization defect [Ducy et al., 1996]. Therefore, the missing mineralization in HOS 58 cells cannot be attributed to any defect in Oc transcription.

Cbfa1 is a recently described osteoblast specific transcription factor essential for the differentiation process of mesenchymal stem cells into mature osteoblasts, and also for maintaining the differentiated cell function during bone formation and remodeling [Mundlos et al., 1997]. Cbfa1-deficient mice show complete lack of bone formation and accompanying calcification defects [Komori et al., 1997; Otto et al., 1997]. In primary human osteoblasts, Cbfa1 mRNA increased during time in culture but only to 3-fold basal levels [unpublished results]. Because we can show an increasing expression of Cbfa1 in HOS 58 cells, the defect in mineralization could not be explained by lack of Cbfa1 gene expression. We suggest that the large increase of Cbfa1 mRNA in HOS 58 cells we observe during culture might be a regulatory phenomenon, probably induced as a consequence of the defective matrix production.

In conclusion, the HOS 58 osteosarcoma cell line represents a differentiated cell line with a highly expressed and physiologically regulated AP expression, and capable of further differentiation in culture. In contrast, gene expression and protein secretion for Oc are dissociated, which could be one possible factor for the missing mineralization in this cell line.

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