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Treatment With 1,25-Dihydroxyvitamin D_3 Reduces Impairment of Human Osteoblast Functions During Cellular Aging in Culture

MARIE KVEIBORG,[1,2] SURESH I.S. RATTAN,[2] BRIAN F.C. CLARK,[2] ERIK F. ERIKSEN,[1] AND MOUSTAPHA KASSEM[1]*

[1] *University Department of Endocrinology and Metabolism, University of Aarhus, Aarhus C, Denmark*

[2] *Laboratory of Cellular Ageing, Department of Molecular and Structural Biology, University of Aarhus, Aarhus C, Denmark*

Adequate responses to various hormones, such as 1,25-dihydroxyvitamin D_3 (calcitriol) are a prerequisite for optimal osteoblast functions. We have previously characterized several human diploid osteoblastic cell lines that exhibit typical in vitro aging characteristics during long-term subculturing. In order to study in vitro age-related changes in osteoblast functions, we compared constitutive mRNA levels of osteoblast-specific genes in early-passage (<50% lifespan completed) with those of late-passage cells (>90% lifespan completed). We found a significant reduction in mRNA levels of alkaline phosphatase (AP: 68%), osteocalcin (OC: 67%), and collagen type I (ColI: 76%) in in vitro senescent late-passage cells compared to early-passage cells, suggesting an in vitro age-related impairment of osteoblast functions. We hypothesized that decreased osteoblast functions with in vitro aging is due to impaired responsiveness to calcitriol known to be important for the regulation of biological activities of the osteoblasts. Thus, we examined changes in vitamin D receptor (VDR) system and the osteoblastic responses to calcitriol treatment during in vitro osteoblast aging. We found no change in the amount of VDR at either steady state mRNA level or protein level with increasing in vitro osteoblast age and examination of VDR localization, nuclear translocation and DNA binding activity revealed no in vitro age-related changes. Furthermore, calcitriol (10^{-8}M) treatment of early-passage osteoblastic cells inhibited their proliferation by $57 \pm 1\%$ and stimulated steady state mRNA levels of AP (1.7 ± 0.1-fold) and OC (1.8 ± 0.2-fold). Similarly, calcitriol treatment increased mRNA levels of AP (1.7 ± 0.2-fold) and OC (3.0 ± 0.3-fold) in late-passage osteoblastic cells. Thus, in vitro senescent osteoblastic cells maintain their responsiveness to calcitriol and some of the observed in vitro age-related decreases in biological markers of osteoblast functions can be reverted by calcitriol treatment. J. Cell. Physiol. 186:298–306, 2001.

1,25-Dihydroxyvitamin D_3 (calcitriol) plays an important role in bone metabolism and it exerts a positive effect on bone mass when administered in vivo (Tilyard et al., 1992). Osteoblasts, the bone forming cells possess nuclear vitamin D receptors (VDR) and are considered the main target for calcitriol action in bone. In vitro, calcitriol inhibits osteoblastic cell proliferation and stimulates the production of several non-collagenous proteins, including osteocalcin and alkaline phosphatase, enhancing thereby the functional activity of mature osteoblastic cells (Beresford et al., 1986; Owen et al., 1990). The biological actions of calcitriol, such as the anti-proliferative effect and transcriptional regulation of differentiated functions of several cell types, are mediated through VDR-dependent pathways (Hannah and Norman, 1994; Hedlund et al., 1996). The critical role of VDR in mediating calcitriol effects on bone has recently been demonstrated by severely impaired bone

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*Correspondence to: Moustapha Kassem, University Department of Endocrinology and Metabolism, Aarhus Amtssygehus, DK-8000 Aarhus C, Denmark. E-mail: mkassem@dadlnet.dk

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formation in VDR knockout mice (Yoshizawa et al., 1997).

An impairment of osteoblast function is thought to be responsible for the general phenomenon of age-related decline in bone mass. Histomorphometric analysis of bone biopsies has demonstrated decreased in vivo bone formation parameters in humans (Cohen-Solal et al., 1991; Brockstedt et al., 1993) and animals (Liang et al., 1992; Quarto et al., 1995) with increasing age. One possible cellular mechanism underlying the age-related decrease of osteoblast functions is impaired responsiveness to various growth factors, cytokines and hormones, such as calcitriol that are important for their optimal performance.

To test the hypothesis of decreased osteoblast responsiveness to calcitriol with increasing age, we employed previously characterized human osteoblastic cell lines that undergo in vitro aging (Kassem et al., 1997). These cell lines are similar to other diploid cell models widely used in research on cellular aging (Rattan, 1995; Campisi, 1996) and they exhibit phenotypic characteristics of in vitro cellular senescence, including a limited proliferative capacity in culture, a progressive decline in the rate of macromolecular synthesis and a dramatic change in morphology (an increase in cell size and spreading, and a changed organization of the cytoskeleton) (Kassem et al., 1997). Also, altered gene expression (Gonos et al., 1998), higher levels of cytoplasmic neutral β-galactosidase activity, and a progressive shortening of mean telomere length (Kveiborg et al., 1999), as well as a progressive decrease in gene expression of the osteoblast-specific transcriptional factor, CBFA-1 (Christiansen et al., 2000) were demonstrated during aging of these cell lines in culture. Here, we report the effects of calcitriol treatment on several biological responses during in vitro aging of human diploid osteoblastic cells.

MATERIALS AND METHODS

Cell culture

A normal diploid human ostoblastic cell line (MK-7398) was established from trabecular bone fragments obtained during orthopedic procedure on a healthy 30-year-old male (after informed consent and approved from the regional ethical committee). Establishing of the human osteoblastic cell lines and their characterization have been previously described (Kassem et al., 1993). In brief, trabecular bone fragments were washed extensively in phosphate buffered saline (PBS), minced and digested with crude collagenase IV (1 mg/L) in Dulbecco's minimal essential medium (DMEM) for 2 h at 37°C. The bone fragments were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (complete medium) (Gibco BRL, Rockville, IL). Cells were allowed to grow from the bone explants and to form a confluent monolayer.

In vitro cell senescence

The MK-7398 cell line was followed throughout its lifespan by serially passaging the cells by trypsinisation at a split ratio of 1:2 or 1:4 once they reached confluence. At each subculturing, the number of cells was counted using a Coulter counter (Coulter Electronics, UK) and the number of population doublings (PDs) was calculated as $\log N/\log 2$, where N is the number of cells in a confluent layer divided by the initial number of cells seeded. Subculturing continued until the cells reached the end of their lifespan, which was evident when they failed to become confluent within four weeks of culturing. The osteoblastic cells were studied at different time points covering their entire lifespan, and we refer to cells with less than 50% lifespan completed as early-passage young cells, cells between 60% and 70% lifespan completed are considered to be intermediate-passage middle-aged cells, and cells with more than 90% lifespan completed are considered late-passage senescent cells.

Assay for short-term growth rate

Osteoblastic cells were seeded in several 24-well plates (Nunc, Roskilde Denmark) at a density of 10^4 cells per well and cultured in complete medium containing either 10^{-8} M calcitriol (kindly provided by Hoffman-La Roche, Denmark) dissolved in 100% ethanol or vehicle (0.1% ethanol). The attachment frequency was determined after 6 h by detaching the cells in three wells and counting the cell number. Cells were subsequently counted as above every day for the first 3–5 days and every second or third day for the following days, until the culture became confluent.

Assay for apoptosis

Osteoblastic cells were seeded on chamber slides at a density of 5×10^4 cells per slide and were cultured for 10 days in the presence of 10^{-8} M calcitriol or vehicle. Cells treated with DNaseI (Promega, Madison, WI) for 10 min or UV-irradiated (60 J/m^2) followed by 24 h growth were used as positive controls. Apoptosis-induced DNA strand breaks were detected using the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) technique (Boehringer Mannheim, Mannheim, Germany) as described by the manufacturer and analyzed under a fluorescence microscope.

RNA preparation and semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR)

Confluent osteoblastic cultures were grown in serum-free medium (with 0.1% BSA) containing various concentrations of calcitriol or vehicle for the times indicated. For RNA isolation, cells were collected in GITC solution (4 M guanidine isothiocyanate, 25 mM sodium acetate (pH 6) and 0.83% β-mercaptoethanol) and total RNA was extracted by a standard protocol employing a CsCl gradient (Davis et al., 1986).

cDNA was synthesized from 4 μg of total RNA in a 20-μL reaction mixture containing 1× reverse transcriptase buffer (5× = 50 mM $MgCl_2$, 250 mM KCl, 250 mM Tris–HCl (pH 8.3), 50 mM dithiothreitol (DTT), 2.5 mM spermidine), dCTP, dGTP, dATP, and dTTP each at 2 mM, 20 U of RNase inhibitor, 8–10 U of AMV reverse transcriptase (all from Promega), 200 pmol of random hexamer primer, and 50 pmol of poly-dT_{15} primer (Boehringer Mannheim). Reaction times were at least 4 h at 42°C.

Aliquots (5%) of the total cDNA were amplified in each PCR in a 20-μL reaction mixture that contained 3–10

TABLE 1. Oligodeoxynucleotide primers used in the PCRa

Gene	Primer concentration (pmol/μl)	Primer sequence (5′–3′)	GenBank accession number	Orientation	Base number	Product size (bp)
GAPDH	3	ACCACAGTCCATGCCATCAC	NM002046	Sense	601–620	452
	3	TCCACCACCCTGTTGCTGTA		Antisense	1033–1052	
Alkaline phosphatase	10	ACGTGGCTAAGAATGTCATC	AB011406	Sense	322–341	475
	10	CTGGTAGGCGATGTCCTTA		Antisense	779–797	
Osteocalcin	10	CATGAGAGCCCTCACA	NM000711	Sense	18–33	310
	10	AGAGCGACACCCTAGAC		Antisense	316–332	
Type 1 procollagen	10	TGACGAGACCAAGAACTG	NM000088	Sense	343–361	599
	10	CCATCCAAACCACTGAAACC		Antisense	934–943	
Vitamin D receptor	10	CGGACACAGCCTGGAGCT	NM000376	Sense	1024–1041	368
	10	ATCTCATTGCCAAACACTTCG		Antisense	1372–1392	

pmol of 5′ and 3′ primer, 1 × PCR buffer (10 × = 500 mM KCl, 100 mM Tris–HCl (pH 9), 1% Triton X-100, dCTP, dGTP, dATP, and dTTP each at 0.2 mM, 1.5 mM $MgCl_2$, 0.2 μL of [α-^{32}P]dATP, 0.4 μL anti-Taq buffer, 0.1 μL anti-Taq (Clonetech, PA), and 0.5 U of Taq polymerase (Perkin-Elmer, Norwalk, CT). Each cDNA sample was run in triplicate for every PCR. The same reaction profile was used for all the primer sets: 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. The PCR primer sequences and concentrations are given in Table 1. PCR reaction products were analyzed by agarose gel electrophoresis, visualized by ethidium bromide staining, and quantified by counting the amount of radioactivity in gel slices (Kassem et al., 1998). GAPDH gene was used as an internal control to correct for possible errors in RNA quantitation and variation in cDNA yield between samples. The PCR products from different experimental conditions were compared only when obtained during the exponential phase of amplification. During this phase, the quantity being measured (i.e., radioactivity) is directly proportional to the amount of material being assayed according to the equation: $N = N_0\ (1 + eff)^n$, where N_0 is the amount of the starting DNA, N is the amount of DNA after n cycles of amplification, and *eff* is the efficiency of amplification, which depends on factors that may be unique for each primer pair and the sequence in between to be amplified (Chelly and Kahn, 1994).

Protein extraction and Western immunoblot analysis

Proteins were isolated from early-passage and late-passage osteoblastic cells using the TRIzol solution (Gibco) as described by the manufacturer (Chomczynski, 1993). The cellular protein content was estimated by the Bio-Rad Protein Assay, based on the Bradford method (Bradford, 1976).

Fifty micrograms protein extracts were resolved by 12.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose filters. The filters were blocked in PBS containing 3% marvel at 4°C overnight and then incubated for 2 h with the 9A7γ rat anti-VDR antibody (Chemicon, CA) diluted 1:1000 in PBS containing 2% marvel. Washings with PBS-tween were repeated three times followed by incubation for 1 h with horse-radish peroxidase (HRP)-conjugated rabbit antibody (Dako, Denmark) against rat-IgG diluted 1:2000 in PBS containing 2% marvel. Finally, the filters were washed three times with PBS-tween and bands were visualized by Enhanced Chemiluminescence (Pierce, Rockford, IL) by incubating with detection reagents for 1 min followed by exposure on a X-ray film.

VDR immunostaining

Early-passage and late-passage osteoblastic cells were grown in complete medium on chamber slides until confluence. The medium was changed to serum-free medium containing either 10^{-8} M calcitriol or vehicle for 3 days. The medium was removed and the cells were fixed with methanol:acetone (1:1), permeabilized with 0.1% Triton X-100 for 10 min, and blocked with 2% BSA for 20 min. The slides were incubated with anti-VDR antibody (9A7γ, Chemicon) 1:100 (diluted in 2% BSA) for 3 h at room temperature, washed with Hank's containing 0.1% Triton X-100 and incubated with FITC-conjugated anti-rat immunoglobulin G (IgG) (Dako) for 30 min. Finally, the slides were washed six times with Hank's containing 0.1% Triton X-100 and the cells were observed by UV-microscopy. A negative control was treated as described except omitting the primary antibody.

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from early-passage and late-passage osteoblastic cells according to the procedure described by Latchman (Latchman, 1993). After 48 h culturing in serum-free medium containing either 10^{-8} M calcitriol or vehicle, cell membranes were disrupted and nuclei were isolated by vortexing the cells in a detergent solution. Extraction of nuclear proteins was obtained by addition of NaCl to a final concentration of 300 mM. The protein concentration was measured using the Bio-Rad Protein Assay, based on the Bradford Method (Bradford, 1976). Complementary synthetic oligonucleotides corresponding to a triple copy of a consensus vitamin D response element (Umesono et al., 1991):

DR3,U: 5′-AGCTTCAGGTCAAGGAGGTCAGA-GAGCT-3′
DR3,L: 5′-CTAGAGCTCTCTGACCTCCTT-GACCTGA-3′,

(DNA Technology, Denmark) were annealed and labeled with [α-^{32}P]dATP (Amersham Pharmacia Biotech, Piscataway, NJ) by a fill-in reaction using the Klenow-fragment of DNA polymerase (New England Biolabs). Five micrograms of nuclear extract was incubated in 20 mM HEPES, 100 mM NaCl, 1 mM DTT, 0.1% NP40, 2 μg poly-(dIdC), and 10% glycerol with or without competitive cold oligonucleotides, antibodies for VDR (9A7γ, Chemicon) or RXRα (ΔN 197, Santa Cruz Biotechnology, Santa Cruz, CA) for 15 min at 4°C. Then 40.000 cpm ^{32}P-DR3 was added to the reaction followed by incubation at room temperature for 30 min. The DR3-protein complexes were resolved by electrophoresis through a non-denaturing 5% polyacrylamide gel. The gels were dried and subjected to autoradiography at −80°C.

Statistics

Dose-response and time-course experiments were analyzed using linear regression analysis. Comparisons between osteoblastic cells at different experimental conditions were performed using paired, two-tailed *t*-test. $P < 0.05$ was considered significant.

RESULTS

Characterization of the MK-7398 cell line

Early-passage MK-7398 cell line exhibited typical characteristics of mature osteoblastic cells, including production of alkaline phosphatase, osteocalcin, and collagen type I. The cells were also able to form a mineralized matrix when incubated with β-glycerophosphate and ascorbic acid at confluence (Kassem et al., 1993).

Effects of calcitriol on biological characteristics of MK-7398 cell line

We examined the effects of calcitriol treatment on MK-7398 cell proliferation. Preliminary studies in early-passage cells were undertaken to determine the optimal concentration of calcitriol to be used. Calcitriol inhibited MK-7398 cell proliferation as demonstrated by [^{3}H]thymidine incorporation assay, in a dose-dependent manner with a maximal inhibitory effect of 44.7 ± 4.7% of control, at a concentration of 10^{-8} M ($P < 0.05$) (data not shown). This concentration of calcitriol was, therefore, used in subsequent experiments. To examine for the mechanism of the antiproliferative effects, we incubated the cells with either calcitriol (10^{-8} M) or vehicle (0.1% ethanol) over a period of 30 days. Figure 1 shows that vehicle-treated control cells exhibited a short lag period followed by an exponential phase of growth during which the cell number increased rapidly (10-fold), before slowing down and reaching a plateau at confluence (11×10^4 cells/well) after about 30 days in culture. The average population doubling time of control cells was 1.8 days. In calcitriol-treated cultures, the duration of the lag period was much longer and the increase in cell number after the lag period was significantly reduced with an average population doubling time of 4.0 days, demonstrating a clear inhibitory effect of calcitriol on osteoblastic cell proliferation rate. However, these inhibitory effects were reversible as evidenced by resumption of normal growth rate in osteoblastic cultures treated for one week only with calcitriol and followed by 3-weeks vehicle-treatment (Fig. 1).

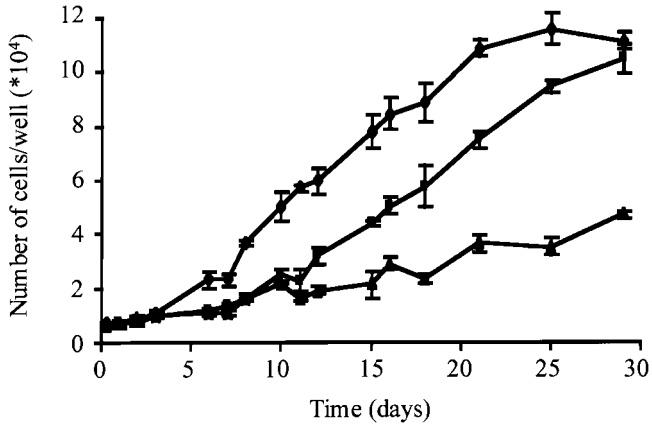

Fig. 1. One-step growth curves of early-passage human trabecular osteoblastic cells showing the number of cells as a function of time in culture. (◆) vehicle-treated cells, (▲) calcitriol- (10^{-8} M) treated cells, and (■) cells treated with calcitriol for 8 days, thereafter vehicle treated. Each point represents mean ± SD (n = 3).

In addition, we examined the effect of calcitriol treatment on cell viability. The number of necrotic and apoptotic cells in osteoblastic cultures treated with calcitriol (10^{-8} M) for 10 days was compared with vehicle-treated cultures after staining with Trypan Blue and by enzymatic in situ labeling of apoptosis-induced DNA strand breaks, using the TUNEL assay (results not shown). Even though calcitriol inhibited osteoblastic cell proliferation after 10 days of treatment, no increase in the number of necrotic cells or apoptotic cells was observed at this time point, indicating that induction of cell death is not the mechanism for the observed inhibition of growth.

We next examined the effects of calcitriol treatment on osteoblast-specific gene expression. Calcitriol treatment increased steady-state mRNA levels of alkaline phosphatase (AP: 3-fold, $P < 0.01$), osteocalcin (OC: 6-fold, $P < 0.001$), and collagen type I (ColI: 2-fold, $P < 0.01$) in a dose- and time-dependent manner, as detected by a semiquantitative RT-PCR assay. The maximal effects of calcitriol were observed at 10^{-8} M concentration after 48–72 h of treatment (results not shown).

Characteristics of MK-7398 cell line during in vitro aging

Figure 2 shows short-term growth of early-passage and late-passage cells and demonstrates a decline in the proliferative capacity of cells with increasing in vitro age. In addition, comparing constitutive mRNA levels of osteoblast specific genes in late-passage cells with those of early-passage cells demonstrated a significant reduction in gene expression of AP (68%, $P < 0.001$), OC (67%, $P < 0.001$), and ColI (76%, $P < 0.001$) (Fig. 3). Other in vitro age-related changes exhibited by this cell line has been reported previously (Kassem et al., 1997; Kveiborg et al., 1999).

In vitro age-related changes in vitamin D receptor (VDR)

Since the effects of calcitriol on osteoblastic cells are mediated through VDR, we first examined the presence

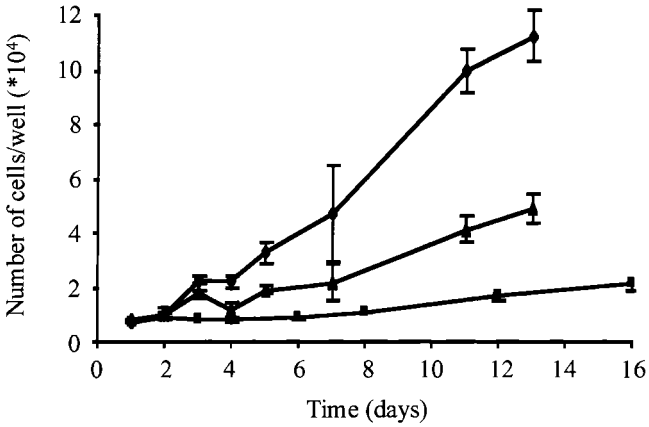

Fig. 2. Effect of calcitriol-treatment on human osteoblastic cell proliferation during in vitro senescence. One-step growth curves of osteoblasts showing the number of cells as a function of time in culture. Early-passage cells (30% lifespan completed) (◆) vehicle-treated, (▲) calcitriol- (10^{-8} M) treated. Late-passage cells (90% lifespan completed) (■) vehicle-treated. Each point represents mean ± SD (n = 3).

and the functional activity of VDR in MK-7398 cell line during in vitro aging. Constitutive levels of mRNA expression and protein production of VDR in early-passage and late-passage osteoblastic cells were determined, employing RT-PCR and Western immunoblot analysis, respectively. After correcting for variation of GAPDH level, we found no significant differences in steady state mRNA VDR levels (Fig. 4a) or protein levels (Fig. 4b) between early-passage and late-passage cells. Furthermore, calcitriol treatment (10^{-8} M) did not change VDR expression levels in either early-passage or late-passage osteoblastic cells (Fig. 4a).

In addition, we examined VDR localization in calcitriol- and vehicle-treated early-passage and late-passage osteoblastic cells using immuno-histochemical staining (Fig. 5). Early-passage untreated osteoblastic cells showed a weak staining for the VDR throughout the cell (Fig. 5a), which upon calcitriol-treatment became concentrated in the nucleus (Fig. 5b). Also, calcitriol treatment increased the fluorescence staining intensity in early-passage cells (Fig. 5b) that may suggest a ligand-induced stabilization of the VDR. Immuno-histochemical staining of late-passage cells demonstrated a positive staining throughout the entire cell, which also upon calcitriol-treatment was found to concentrate in the nucleus (Fig. 5c,d).

Transcriptional regulation by VDR requires binding of VDR/VDR-homodimers or VDR/retinoic X receptor (RXR)-heterodimers to vitamin D responsive elements (VDREs) in the promoter region of target genes. We next compared the binding of nuclear proteins from early-passage and late-passage osteoblastic cells to a consensus VDRE nucleotide-sequence using electrophoretic mobility shift assay (EMSA). Figure 6a shows the complexes formed by binding of nuclear proteins from untreated vs. calcitriol (10^{-8} M) treated early-passage and late-passage osteoblastic cells. Complex formation is clearly increased upon addition of the ligand in both early-passage and late-passage cells. Two bands (arrows 1 and 2) represent specific complexes as demonstrated by complete displacement with a specific competitor (non-radiolabled DR3 oligonucleotide probe) (Fig. 6a, lanes 4, 8; Fig. 6b, lanes 2, 6). No competition was observed with an unrelated oligonucleotide probe (not shown). The complexes seem to contain VDR since addition of an antibody against VDR, which is known to bind an epitope in the DNA binding domain, weakens the binding to the DR3 oligonucleotide probe (Fig. 6b, lanes 3,7). Furthermore, addition of an antibody recognizing RXR-α, -β and -γ changes the appearance of complex formation (Fig. 6b, lanes 4,8), and after a long exposure of the film, a weak supershifted band was observed. However, in order to be able to compare complex formation in early-passage versus late-passage osteoblastic cells less exposure time were required and thus, the supershift is not visible. No significant difference between complex formation in early-passage versus late-passage cells was detected, suggesting that VDR DNA binding activity is unaffected by in vitro aging.

In vitro age-related changes in the effect of vitamin D on osteoblast functional markers

Having established the presence of intact VDR system during in vitro aging, we examined the regulation of osteoblast-specific genes by calcitriol treatment. Figure 3 shows the effects of calcitriol (10^{-8} M) treatment for 72 h on steady state mRNA levels of markers of osteoblast functions in early-passage, intermediate-passage and late-passage cells. Calcitriol-treatment (10^{-8} M) increased mRNA levels of AP (1.7 ± 0.1-fold in early-passage cells; 1.7 ± 0.2-fold in late-passage cells), and OC (1.8 ± 0.2-fold in early-passage cells; 3.0 ± 0.3-fold in late-passage cells) ($P < 0.05$) (Fig. 3). Calcitriol-induced increase in mRNA levels of OC in late-passage osteoblastic cells reached a level comparable to those of early-passage untreated osteoblastic cells. However, the effects of calcitriol treatment on ColI were dependent on in vitro cell age with some decrease in early-passage osteoblastic cells (0.62 ± 0.3-fold) and an increase of variable magnitude in intermediate-passage (1.34 ± 0.1-fold) and early-passage cells (1.36 ± 0.1-fold), but these changes were not statistically significant. The calcitriol-induced changes in mRNA levels of AP and ColI in late-passage cells were, however, lower than the constitutive mRNA levels detected in untreated early-passage osteoblastic cells ($P < 0.01$).

DISCUSSION

Decreased bone formation at the tissue level has been demonstrated in the aged skeleton of humans (Brockstedt et al., 1993) and in rodents (Liang et al., 1992; Quarto et al., 1995), and it can be caused by either reduced number of recruited osteoblast precursors and/or reduced functional activity of mature osteoblasts during bone formation phase of the skeletal remodeling cycles (Parfitt, 1990). In order to study the factors responsible for age-related decrease in functional activity of mature osteoblastic cells, we employed a normal human diploid osteoblastic cell line that undergoes in vitro aging and expresses characteristics of mature osteoblastic phenotype (Kassem et al., 1997). We found a dramatic in vitro age-related decline in the consititutive mRNA levels of biological markers of osteoblast functions: AP, OC, and ColI in culture. The

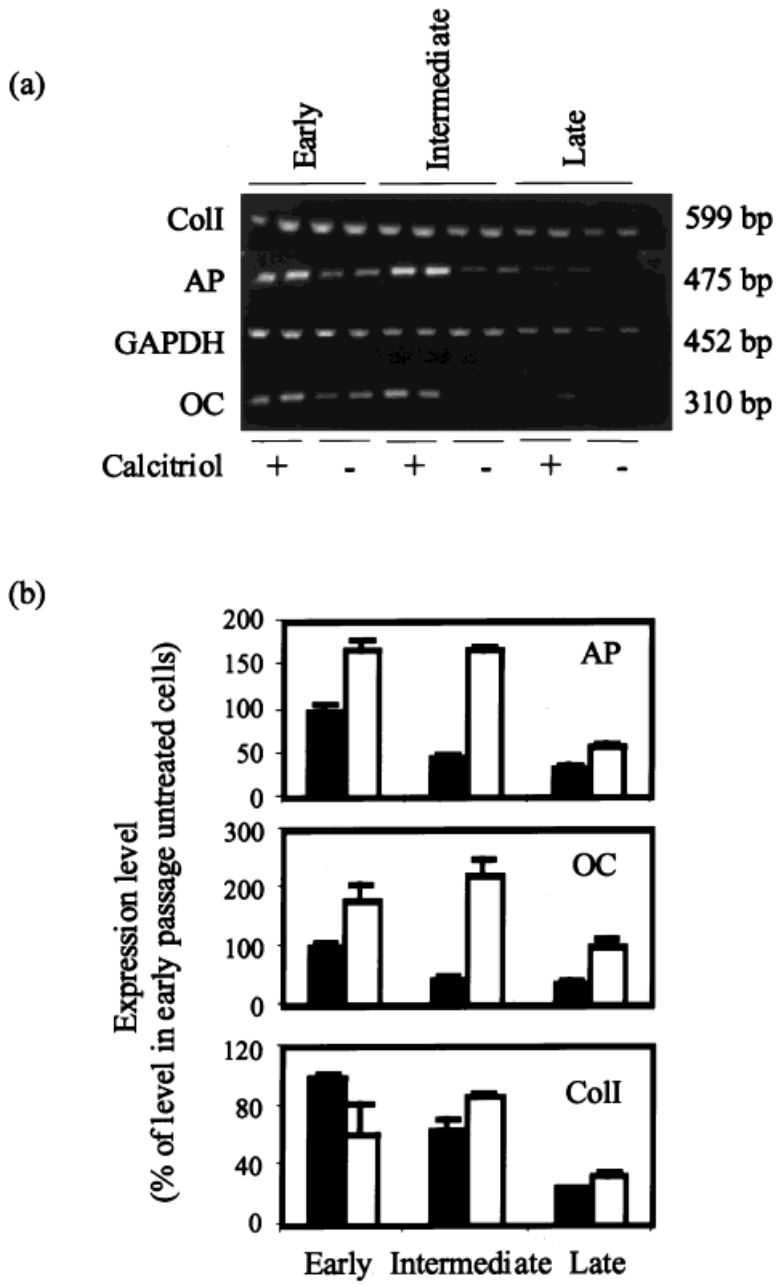

Fig. 3. In vitro age-related changes in the expression level of alkaline phosphatase (AP), osteocalcin (OC), and collagen type I (ColI) analyzed by RT-PCR assay. RNA was isolated from early-passage (40% lifespan completed), intermediate-passage (70% lifespan completed) and late-passage (100% lifespan completed) human osteoblastic cells treated with (+) or without (−) calcitriol (10^{-8} M) for 72 h. (**a**) Reaction products visualized on ethidium bromide stained agarose gels. The sizes of PCR products are indicated. (**b**) Relative percentage expression of mRNA levels in calcitriol-treated (open bars) and vehicle-treated (dense bars) early-passage, intermediate-passage, and late-passage osteoblastic cells. The data are corrected for GAPDH levels and presented as mean mRNA levels ± SD (n = 3) as a percentage of levels observed in early-passage vehicle-treated cells (Rel. expression = 100%).

products of these genes are important for bone matrix formation (ColI), matrix maturation, and mineralization (AP, OC) (Owen et al., 1990). While we did not study the effect of in vitro age on the ability of osteoblastic cells to form a bone matrix in vitro, the decreased production of the osteoblast-specific genes indicate an impairment of their functional abilities. The data obtained from our cell culture model corroborate previous findings in cultured human osteoblastic cells that were obtained from donors of different ages. A negative correlation between donor age and the osteoblastic production of collagen type I, osteocalcin, other extracellular matrix proteins as well as alkaline phosphatase enzyme activity in culture was reported (Chavassieux et al., 1990; Fedarko et al., 1992; Sutherland et al., 1995). Thus, the decreased osteoblastic functions observed in

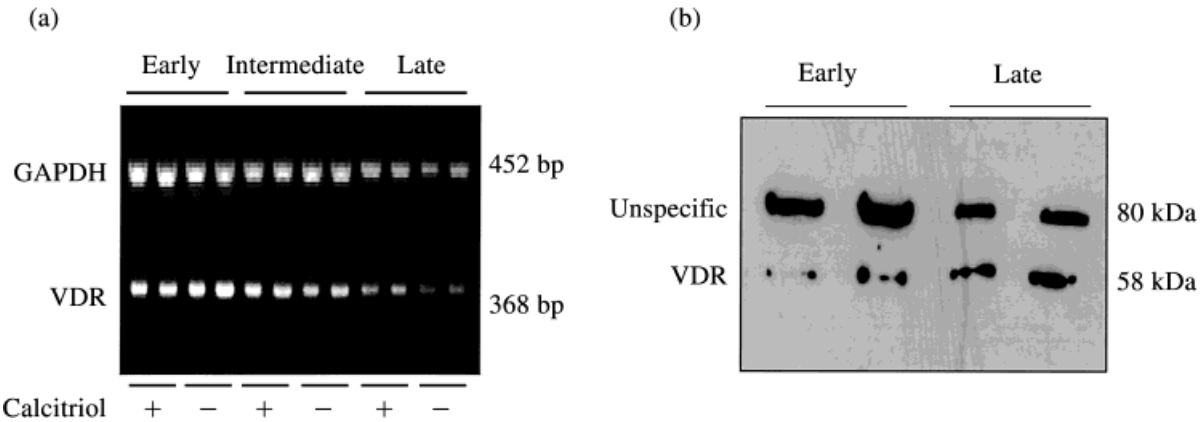

Fig. 4. Analysis of vitamin D receptor (VDR) expression, assessed by (**a**) RT-PCR in calcitriol- (10^{-8} M) (+) or vehicle- (−) treated human osteoblastic cells at three points covering the entire lifespan (early-passage, intermediate-passage, late-passage). PCR products in duplicate are visualized by ethidium bromide stained agarose gels and the sizes are indicated on the right. (**b**) Western blot in early-passage and late-passage osteoblastic cells, showing two bands, one unspecific (85 kDa) and VDR (60 kDa). Each sample is run in duplicate.

vivo in humans could be demonstrated reproducibly by in vitro cultured cells.

Consistent with previous findings in osteoblastic cell cultures (Beresford et al., 1986; Scharla et al., 1991), calcitriol inhibited cell proliferation in MK-7398 cell line. The inhibitory effects of calcitriol on cell growth seem to be a general phenomenon that has been reported in several cell types (Bikle, 1992; Walters, 1992). The anti-proliferative effects of calcitriol are mediated through VDR and via a genomic signaling pathway (Hedlund et al., 1996). We found that the inhibitory effect of calcitriol on osteoblastic cell proliferation was not mediated through induction of apoptosis or cell necrosis. We have not studied the effects of calcitriol treatment on cell cycle dynamics in our culture, but the reversibility of its effects on cell growth may be explained by changes in production of cell cycle genes, such as pRb, p21, and CDK2 (Liu et al., 1996; Zhuang and Burnstein, 1998) or transcription factors (Hannah and Norman, 1994) known to regulate cell proliferation. Alternatively, calcitriol can inhibit cell proliferation *indirectly*. It was reported that calcitriol increases gene expression and production of insulin-like growth factor binding protein (IGFBP)-4 known to inhibit osteoblastic cell proliferation (Scharla et al., 1991).

Decreased responsiveness to hormones and growth factors has been suggested to be responsible for the decreased functional activity of in vitro senescent cells (Phillips et al., 1984; Cristofalo and Pignolo, 1996). This effect may be caused by defective receptor-mediated

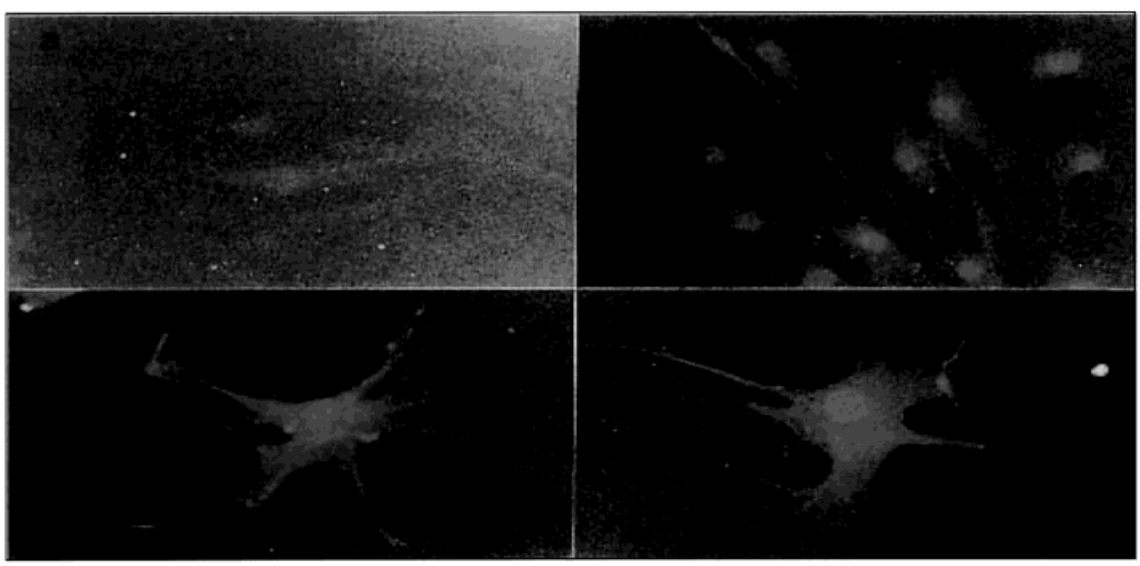

Fig. 5. Immunolocalization of the vitamin D receptor (VDR) in human osteoblastic cells during in vitro senescence. Immunohistochemical staining using a primary monoclonal anti-VDR antibody and a FITC-conjugated secondary antibody, and subsequent examination by UV-microscopy in osteoblast cultures. Early-passage (45% lifespan completed) (**a**) untreated cells, (**b**) calcitriol-treated cells. Late-passage (>95% lifespan completed) (**c**) untreated cells, (**d**) calcitriol-treated cells. Pictures are taken with equal exposure time and a microscopic magnification × 40.

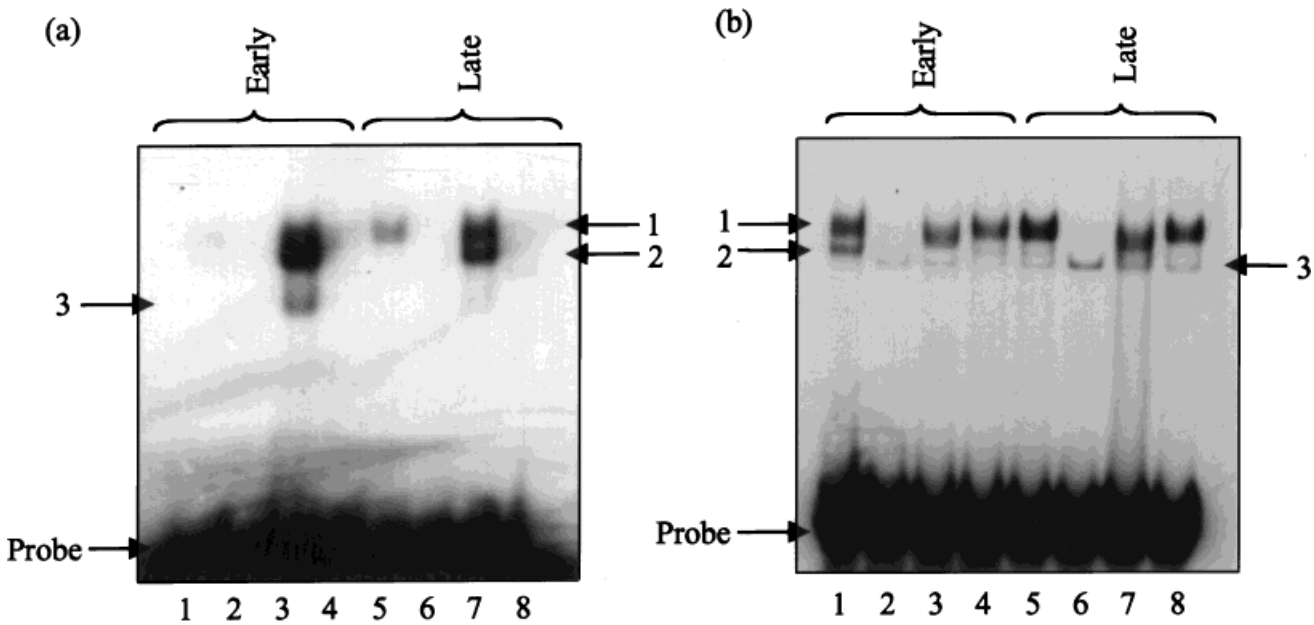

Fig. 6. Electrophoretic mobility shift assays comparing DNA binding activity of nuclear proteins from early-passage and late-passage osteoblastic cells. Bands 1 and 2 indicate specific binding to the DR3 probe, whereas band 3 represents unspecific complex formation. (**a**) Lanes 1,2: early-passage untreated cells; lanes 3,4: early-passage calcitriol-treated cells; lanes 5,6: late-passage untreated cells; lanes 7,8: late-passage calcitriol-treated cells. In lanes 2, 4, 6, and 8 competitive DR3 oligo was added. (**b**) Lanes 1–4: early-passage calcitriol-treated cells; lanes 5–8: late-passage calcitriol-treated cells. Competitive DR3 oligo was added in lanes 2 and 6; VDR antibody was added in lanes 3 and 7; RXRα antibody was added in lanes 4 and 8.

signaling. Previous studies on age-related changes in VDR reported the presence of an age-related decrease in intestinal VDR levels and also age-related post-receptor changes leading to intestinal vitamin D resistance (Ebeling et al., 1992; Wood et al., 1998). We found no detectable differences in the amount of VDR mRNA or protein between early-passage and late-passage osteoblastic cells. Also, analysis of VDR localization and ligand-induced nuclear translocation was similar in both early-passage and late-passage osteoblastic cells. Furthermore, VDR nuclear DNA binding as demonstrated by the electrophoretic mobility shift assay was efficient in both early-passage and late-passage cells. These results indicate that the osteoblasts maintain a functional VDR system during in vitro aging and the observed age-related VDR changes in the intestine is not a generalized phenomenon.

In vitro senescent osteoblastic cells maintained their responsiveness to calcitriol as demonstrated by increased OC and AP mRNA levels. The calcitriol-induced increase in OC mRNA in vitro senescent late-passage cells reached the constitutive levels of untreated early-passage cells, indicating the ability of calcitriol to abolish some of the in vitro age-related impairment of osteoblast functions. Similar to our findings, Wong et al. (1994) reported maintenance of calcitriol-induced increase in AP production by osteoblastic cells isolated from donors of different ages and in patients with osteoporosis. Thus, our results do not support the hypothesis that age-related impaired osteoblast functions is due to the presence of calcitriol resistance at the levels of the osteoblastic cells. However, our findings do not exclude the presence of impaired osteoblast responsiveness to other growth factors and cytokines or the presence of an intrinsic age-related defect in osteoblast functions (Parfitt, 1990; Kveiborg et al., 1999). Also, it is possible that decreased cell recruitment from the osteoblast precursor pool is the rate-limiting step during bone formation in vivo (Parfitt, 1990). These alternative possibilities remain to be addressed in experimental models.

Our findings, that calcitriol treatment of in vitro aged osteoblastic cells can increase their gene expression of osteoblast specific genes necessary for bone formation, may be relevant for understanding the in vivo cellular effects of calcitriol on age-related bone loss. Several clinical studies have demonstrated that calcitriol treatment stabilizes bone-mass and can prevent osteoporotic fractures (Tilyard et al., 1992; Eastell, 1998). The positive effects of calcitriol on bone mass has usually been attributed to its effects on increased intestinal calcium absorption and inhibition of age-related secondary hyperparathyroidism (Riggs and Melton, 1986; Eastell, 1998). Our findings suggest the presence of an additional mechanism of direct anabolic effects of calcitriol on the aged osteoblasts.

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