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Hydrocortisone Increases the Rate of Differentiation of Cultured Human Osteoblasts

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Abstract Reduced bone formation is the main finding in glucocorticoid-induced osteoporosis. The aim of this study was to determine whether differentiation of cultured human osteoblasts is inhibited by high concentrations of hydrocortisone. We measured the levels of mRNAs for three markers of cellular differentiation, type 1 collagen (COL1), alkaline phosphatase (ALP), and osteocalcin (OC), in four lines of human osteoblasts from female donors cultured with doses of hydrocortisone from 0 μM to 4 μM. The change in ALP/COL1 mRNA ratio over a given time was used to determine the average rate of differentiation of the cells in a culture. Although basal expression profiles and their changes with time were different for the different cell lines, all cell lines showed a dose-dependent rise in the rate of increase of ALP mRNA relative to COL1 mRNA. However, increase in OC mRNA with time, seen here only in young donor hOBs, was significantly inhibited by 4 μM hydrocortisone, indicating that hydrocortisone can inhibit OC expression while promoting cellular differentiation. The data suggest that increasing concentrations of glucocorticoid, including concentrations similar to plasma levels in patients receiving oral glucocorticoid therapy, increase the rate of cellular differentiation. J. Cell. Biochem. 91: 594–601, 2004.

Key words: glucocorticoid; bone cells; proliferation; differentiation; gene expression

Physiological concentrations of glucocorticoids are essential for the differentiation and function of bone cells but higher concentrations have deleterious effects on bone metabolism [Canalis, 1996]. Hypercortisolism in Cushing's disease is associated with bone loss that is at least partially reversible by surgical cure [Hermus et al., 1995]. Therapeutic use of glucocorticoids is a major cause of osteoporosis and fragility fractures. Van Staa et al. [2000] concluded, after analysis of a study that included a quarter of a million steroid users, that the risk of vertebral and proximal femoral fractures increases in direct proportion to the daily dose of steroid. Furthermore, risk begins to increase as early as 3 months after therapy is begun and decreases rapidly when medication is stopped.

Histomorphometry of bone biopsies taken from glucocorticoid-treated patients has shown reduction in bone formation, mainly in cancellous bone [Dempster, 1989; Rubin and Bilezikian, 2002]. Trabecular wall thickness in bone biopsies from steroid patients was even lower than that seen in patients with postmenopausal osteoporosis [Chappard et al., 1996; Ishida and Heersche, 1998; Carbonare et al., 2001]. The mechanisms involved in this reduction in bone formation are not clearly understood; decreased osteoblast replication and differentiation, and increased mature osteoblast apoptosis have been suggested [Canalis and Delany, 2002].

Osteoblast precursors originate from pluripotent mesenchymal stem cells that are influenced by bone morphogenetic proteins and other factors to become committed to the osteoblastic lineage. The transcription factor Runx2 activates genes such as type I collagen (*COL1*), alkaline phosphatase (*ALP*), and osteocalcin (*OC*). In cultured cells, ALP mRNA expression is coupled to the down-regulation of proliferation. ALP mRNA is low during proliferation, rises rapidly during the period of matrix maturation and then starts to fall, whereas the level of COL1 mRNA increases during proliferation and

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differentiation [Lian et al., 1999]. OC mRNA expression is not coupled to proliferation but remains low until the onset of mineralization.

Osteoblast cultures are not homogeneous but contain cells at different stages of differentiation. During the early and intermediate stages of differentiation, the ALP/COL1 mRNA ratio increases as more cells cease dividing and become osteoblasts. The differentiation rate, i.e., the number of precursor cells that differentiate in each cell cycle, is an important factor in determining tissue growth rate [Arendt, 2000]. Until ALP expression falls with mineralization, faster differentiation of an osteoblast culture will be reflected in a greater rate of increase in ALP/COL1 mRNA ratio.

We hypothesized that hydrocortisone, a known promoter of differentiation in cultured osteoblasts, does not inhibit osteoblast differentiation at high concentrations but rather increases the rate of differentiation. In spite of the known heterogeneity of osteoblast cultures [Gronthos et al., 1999; Aubin and Heersche, 2000], increased hydrocortisone concentrations should result in greater increases in ALP/COL1 ratios over a given time in cells cultured with increased hydrocortisone concentrations. The expression of OC may not follow the same pattern since Viereck et al. [2002] reported differential regulation of Runx2 and OC in cultured hOBs by dexamethasone. A >4-fold increase in Runx2 expression over time was accompanied by 68% inhibition of OC.

In order to test our hypothesis, we used quantitative real-time RT-PCR to measure ALP, COL1, and OC mRNAs in young and old donor hOBs from different anatomical sites during 8 days culture with increasing doses of hydrocortisone. For all cell lines, changes in ALP/COL1 ratios during the culture period were calculated for each hydrocortisone dose used.

MATERIALS AND METHODS

Cells

Four lines of hOBs, two from young (A and B) and two from older (C and D) female donors, were used in these experiments. Cell line B, from a 1-year-old (anatomical site unknown) and C, from hip bone of a 67-year-old, were supplied by two companies (BioWhittaker UK Ltd., Wokingham and Promocell GmbH, Heidelberg, Germany). The other cells lines were isolated in our laboratory by sequential enzyme digestion of waste bone from surgical procedures after local ethical committee approval and informed consent had been obtained. Cell line A was isolated from an accessory digit of a 3-month-old and D from vertebral bone of a 67-year-old. All cell lines were used at passage seven or eight.

Culture Conditons

Cells were cultured in plastic tissue culture flasks containing McCoy's 5A (modified) medium supplemented with 100 μM long-life ascorbic acid (WAKO, Alpha Labs, Eastleigh, Sussex, UK), 10% pooled male human AB serum, 200 mM glutamine, and antibiotics. The 25 cm^2 flasks were seeded with 5×10^5 cells per flask, a number that ensured that all cultures except young donor hOBs (hand) remained sub-confluent throughout the experiment. On day zero of the experiment, 4 μM β-cyclodextrin (carrier) was added to two flasks of control cells, and 0.2 μM and 4 μM cyclodextrin-encapsulated hydrocortisone were added to two flasks at each concentration. The 0.2 μM hydrocortisone is in the normal adult physiological range in serum and 4 μM is a typical plasma level after oral glucocorticoid administration [Ishida and Heersche, 1998]. The medium was changed every 2 days. One flask of cells at each hydrocortisone concentration was harvested at 2 days and the remaining flasks were harvested at 4 days (cell line A) or 8 days. Day 2 was chosen for the baseline so that changes in ALP/COL1 mRNA ratio with time would reflect the differentiation rate of the cells in a given hydrocortisone concentration and not transient changes in gene expression due to alterations in hydrocortisone concentration. Since all cultures were harvested before they became confluent, the most proliferative cells, from the youngest donor, had to be harvested at an earlier time point.

RNA Extraction

Poly(A) RNA was extracted from cultured cells using the MicroPoly(A)Pure^{TM} kit (Ambion (Europe) Ltd., Huntingdon, UK) according to the manufacturer's instructions.

Preparation of RNA Standards

PCR products for COL1A1, ALP, OC, and glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) were made by RT-PCR of human endometrial, OB, or tendon RNA. Primer sequences are shown in Table I. The PCR products were cloned into the T-tailed vector pCR II-TOPO (Invitrogen Ltd., Paisley, UK) which has opposing SP6 and T7 RNA polymerase promotor sites. BamHI restriction sites were added to the forward primers so that the orientation of each insert could be determined by cutting the plasmids with BamHI. Plasmids that contained inserts suitable for transcription with SP6 RNA polymerase were selected and cut with Not1. RNA transcripts were purified using Microspin S-300 columns (Amersham Biosciences UK Ltd., Little Chalfont) after treatment with DNase1 to remove plasmid DNA.

RT-PCR Using GeneAmp 5700 SDS

One-step RT-PCR reagents (Applied Biosystems, Warrington, UK) were used in the Gene Amp 5700 SDS. Primer Express™ software was used to design the primers and probes. All primer pairs were chosen to include introns in the gene sequences. Probes were chosen to span intron–exon boundaries so that they were unable to hybridize to genomic DNA at the annealing temperature of the PCRs. The sequences are shown in Table I. A standard curve was included in each assay so that the overall efficiency of the assay could be calculated.

Relative Quantification of Gene Expression

mRNA levels were determined using the comparative threshold-cycle (C_T) method [Fink et al., 1998]. First, the amount of target mRNA in each sample was normalized to the amount of housekeeper mRNA (GAPDH) to give $\Delta C_T \left(C_{T\,\mathrm{target}} - C_{T\mathrm{GAPDH}}\right)$.

Secondly, normalized target mRNAs in the samples were compared to the value for cell line A cultured for 2 days without added hydrocortisone (calibrator) using the formula

mRNA expression relative to cell line A cultured for 2 days without added hydrocortisone $= 2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T = \Delta C_{T\,\mathrm{sample}} - \Delta C_{T\,\mathrm{calibrator}}$ assuming that the efficiencies of the PCR reactions were close to one. The efficiency of each assay was calculated using the formula $E = 10^{-1/S} - 1$ where $S =$ slope of the standard curve.

The use of cell line A cultured for 2 days without added hydrocortisone as calibrator

TABLE I. Primers and Probes Used for Cloning and RT-PCR

Gene	Forward primer	Reverse primer	Probe
Cloning primers			
GAPDH	TGAAGGTCGGAGTCAACGGATTTG	GTTGGTGGTGCAGGAGGCATTGCT	
COL1A1	CCCCCTCCCCAGCCACAAAGA	TCTTGGTCGGTGGGTGACTCT	
ALP	GTACTGGCCATTGGCACCCTG	GGCCTTCACCCCACACAG	
OC	CTCGCCCTATTGGCCCTG	GATAGGCCTCCTGAAAGCCAT	
GeneAmp 5700 SDS primers and probes			
GAPDH	TTTTAACTCTGGTAAAGTGGATATTGTTG	TGACGGTGCCATGGAATTT	ATTGACCTCAACTACATGGTTTACATGTTCCAATATG
COL1A1	CGCACGGCCAAGAGGAA	CATGGTACCTGAGGCCGTTCT	CCAAGACGAAGACATCCCACCAATCACC
ALP	CAGGCTGGAGATCGACAAGTTC	GGACCTGGCATTGGTGTT	CCTTCGTGGCCCTCTCCAAGACGT
OC	AGCAAGGTGCAGCCTTTGT	ACAGGTAGCGCCTGGGTCT	CTTCACTACCTCGCTGCCCTCCTGCTT

for all samples allowed comparison of specific mRNA levels in the different cell lines.

Changes in levels of ALP mRNA relative to COL1 mRNA with time were calculated by a similar method. ΔC_T was now $C_{TALP} - C_{TCOL1}$. Normalization of the values to a housekeeper mRNA is not needed in this case. The change in the ALP/COL1 mRNA ratio with time for each hydrocortisone concentration was determined using the formula change in ALP/COL1 mRNA ratio day8/day2 (day4/day2 for A)$= 2^{-\Delta\Delta C_T}$ where $\Delta\Delta C_T = \Delta C_{T\ day8(4)} - \Delta C_{T\ day2}$

Statistical Analysis

Expression profiles and their changes with time in the different cell lines cultured in basal medium containing no added hydrocortisone were compared. mRNA levels in cells cultured with hydrocortisone were compared to levels in controls without added hydrocortisone at the same time point and increases with time were calculated for each hydrocortisone dose. mRNA levels in the different cell lines cultured with hydrocortisone were also compared. RNAs were tested using four replicates and the data analyzed using the Satterthwaite approximate test for unequal variances based on the t-distribution to compare the means of the samples [Armitage and Berry, 1994].

RESULTS

Basal expression profiles and their changes with time were different for the different cell lines. In basal medium containing no added hydrocortisone, baseline (day 2) ALP mRNA was lower in A and B (young donor hOBs) than in C and D (old donor hOBs). The maximum difference was 18-fold between A and C (Fig. 1). ALP mRNA in cells grown in basal medium increased or remained the same between days 2 and 4 or 8 in A and B but decreased in C and D. Baseline COL1 mRNA showed less variation between cell lines than baseline ALP mRNA with a maximum 3-fold difference between C and D (Fig. 2). In basal medium, the level of COL1 mRNA increased with time in A, B, and D but decreased in C. OC mRNA levels on day 2 were similar in all cell lines grown in basal medium (Fig. 3) but increased with time only in A and B. Thus, when cultured at passage seven or eight in medium without added hydrocortisone, lines C and D showed no increases in ALP and OC mRNAs and only D showed an increase in COL1 mRNA. However, these cell lines from older donors are clearly osteoblastic since they are able to mineralize under appropriate culture conditions. When cell lines B (young donor hOBs) and C (old donor hOBs) were cultured on collagen in a medium containing 0.2 μM hydrocortisone and 7.5 mM β-glycerophosphate, C

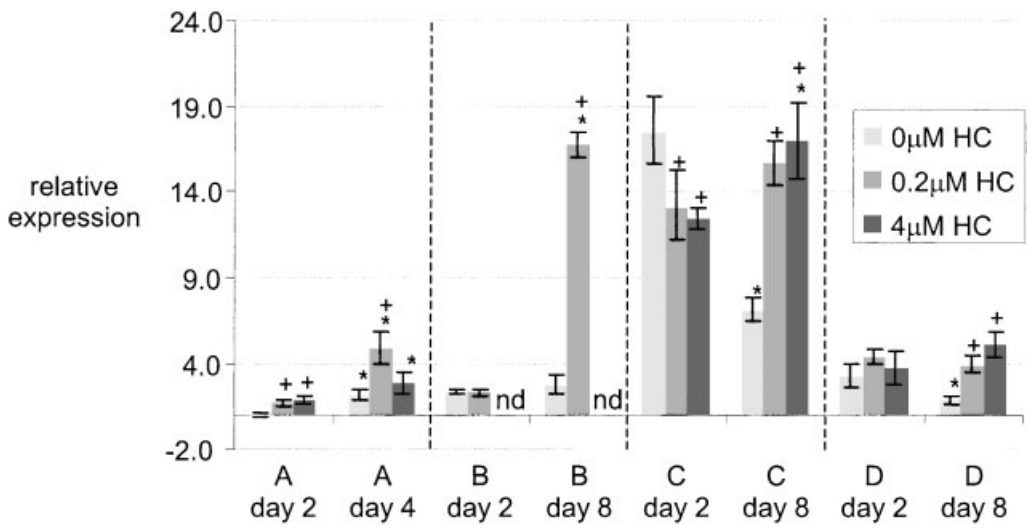

Fig. 1. Alkaline phosphatase (ALP) mRNA expression in hOBs cultured with increasing concentrations of hydrocortisone. Values are expressed relative to the baseline level in cell line A cultured without added hydrocortisone. Mean values ± SD for four replicates in a representative experiment are shown. +, Indicates a significant change ($P < 0.05$) from control cells without added hydrocortisone; *, indicates a significant change ($P < 0.05$) between days 2 and 4 or 8; nd, indicates not done.

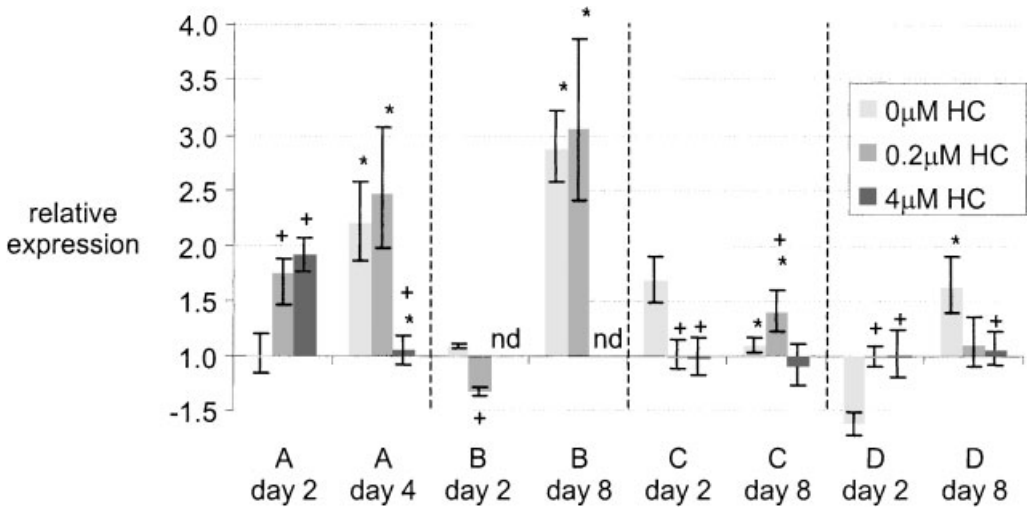

Fig. 2. Type 1 collagen (COL1) mRNA expression in hOBs cultured with increasing concentrations of hydrocortisone. Values are expressed relative to the baseline level in cell line A cultured without added hydrocortisone. Mean values ± SD for four replicates in a representative experiment are shown. +, Indicates a significant change ($P < 0.05$) from control cells without added hydrocortisone; *, indicates a significant change ($P < 0.05$) between days 2 and 4 or 8; nd, indicates not done.

formed mineralized nodules in 1 week whereas B did not [Ireland et al., 2002].

ALP mRNA was increased by hydrocortisone in A on day 2 and in all cell lines on day 4 or 8. hOBs B and C cultured in 0.2 μM hydrocortisone had similar levels of ALP mRNA expression on day 8 in spite of their >5-fold difference on day 2. With 0.2 μM hydrocortisone, the level of ALP mRNA increased with time approximately 3-fold in A and 7-fold in B but did not change in C or D. ALP mRNA increased with time approximately 1.5-fold in A, C, and D when 4 μM hydrocortisone was added to the culture medium.

On day 2, levels of COL1 mRNA were increased by hydrocortisone in A and D and decreased in the other cell lines. On day 4 or 8, COL1 mRNA levels were higher in A and B than in C and D. The 0.2 μM hydrocortisone increased

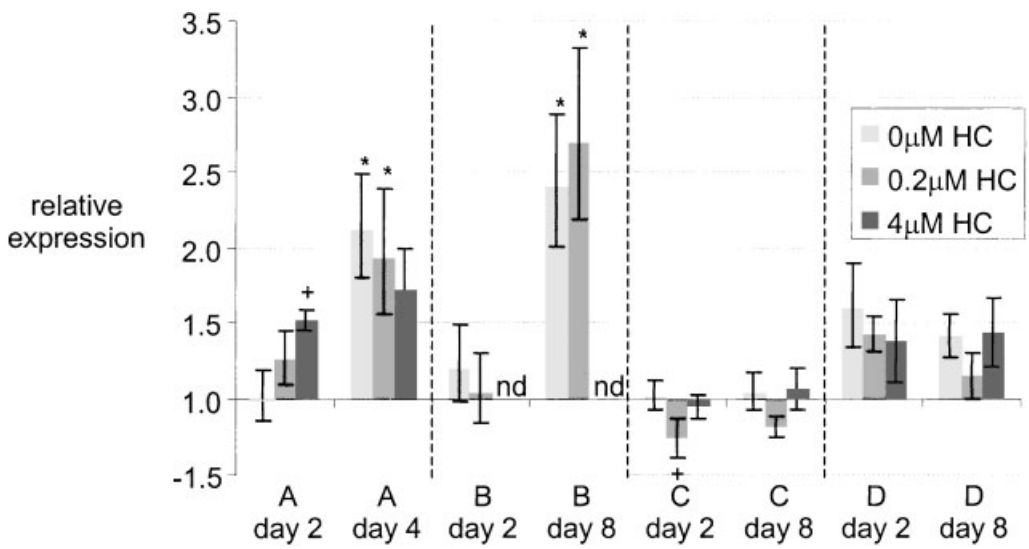

Fig. 3. Osteocalcin (OC) mRNA expression in hOBs cultured with increasing concentrations of hydrocortisone. Values are expressed relative to the baseline level in cell line A cultured without added hydrocortisone. Mean values ± SD for four replicates in a representative experiment are shown. +, Indicates a significant change ($P < 0.05$) from control cells without added hydrocortisone; *, indicates a significant change ($P < 0.05$) between days 2 and 4 or 8; nd, indicates not done.

COL1 mRNA with time in all cell lines while 4 μM hydrocortisone decreased COL1 mRNA 1.8-fold in A but did not change the level of this mRNA in C and D.

Baseline levels of OC mRNA were increased by hydrocortisone only in A. On day 4 or 8, OC mRNA levels were higher in A and B than in C and D. With 0.2 μM hydrocortisone, OC mRNA increased with time in A and B (1.6-fold in A and 2.5-fold in B) but not in C and D. The 4 μM hydrocortisone produced no change in OC mRNA with time in any of the cell lines.

Changes in ALP/COL1 mRNA with time are shown in Figure 4. All four cell lines showed a hydrocortisone dose-dependent rise in the rate of increase of ALP mRNA relative to COL1 mRNA.

DISCUSSION

Although baseline levels of COL1 and ALP mRNAs were different in the different cell lines, all the cell lines showed a hydrocortisone dose-dependent rise in the rate of increase of ALP mRNA relative to COL1 mRNA. However, under the culture conditions used, OC mRNA, which normally increases with differentiation, increased with time only in young donor hOBs and this increase was significantly inhibited in cell line A by 4 μM hydrocortisone. The data suggest that increasing concentrations of glucocorticoid increase the rate of cellular differentiation while inhibiting OC expression.

The osteoblast cultures used here came from different skeletal sites of various donors, young and old. Unfortunately we have no data on the effect of passage on gene expression in these cells. However, we have measured ALP mRNA expression in a number of hOB lines at passage eight or nine and in two osteosarcoma lines cultured for 2 days in medium containing 0.2 μM HC. Young donor hOBs (A and B), TE85 and MG63 had the lowest ALP mRNA levels while five lines of old donor hOBs (including C and D) had levels 2.5–7.6-fold higher than A. Progressive reduction with age and passage number of cultured rat and human osteoprogenitor self-renewal has been reported in recent publications [Banfi et al., 2002; Bellows et al., 2003; Schecroun and Delloye, 2003]. Thus, increasingly differentiated cell populations are used to initiate each subculture with cells from older donors being more differentiated than those from younger donors at an equivalent passage. Reduced osteoprogenitor self-renewal, reflected in the slower proliferation of old donor hOBs, probably explains the higher ALP mRNA levels in C and D.

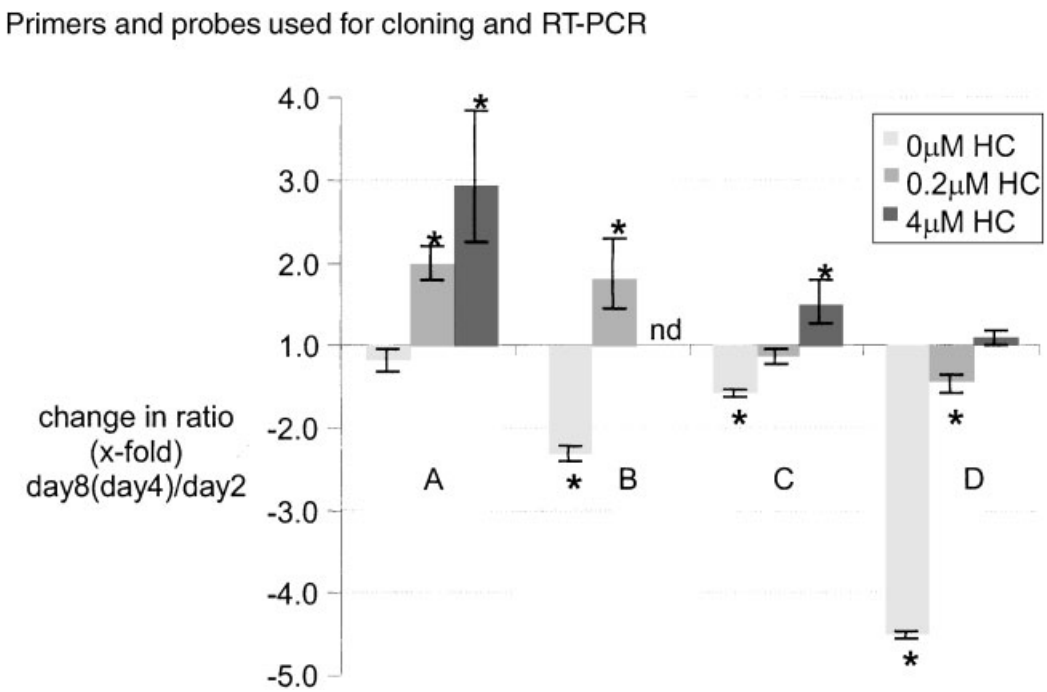

Fig. 4. Changes in ALP/COL1 mRNA ratios in hOBs cultured with increasing doses of hydrocortisone. Mean values ± SD for four replicates in a representative experiment are shown. Significant changes ($P < 0.05$) are marked with an asterisk.

Previous experiments using human bone marrow stromal cells cultured with glucocorticoids to investigate their effects on osteoblast differentiation demonstrated that high concentrations of dexamethasone inhibit osteoblast proliferation but not differentiation and further maturation [Jaiswal et al., 1997; Walsh et al., 2001].

Culture of the osteoblast lines in medium without added HC resulted in decreasing ALP/COL1 mRNA ratios with time in all cell lines (Fig. 4). The level of cortisol in the medium (15 nM from the human serum component) is less than the normal plasma concentration in both children and adults. The 0.2 μM hydrocortisone, equivalent to the normal adult plasma concentration, resulted in increases in ALP/COL1 mRNA ratios with time in young donor hOBs compared to small decreases in old donor hOBs. However, levels of plasma cortisol are generally lower in children than in adults [Tornhage, 2002]. The 4 μM hydrocortisone, a typical plasma level after oral glucocorticoid administration, resulted in a 3-fold increase in ALP/COL1 mRNA ratio with time in young donor hOBs and smaller increases in old donor hOBs.

The changes in ALP and COL1 mRNAs provided sufficient information to measure rates of cellular differentiation. Assuming the changes in mRNA levels reflect the changes in the cognate proteins, the data suggest a model in which glucocorticoid-induced enhanced osteoblast differentiation reduces bone formation by limiting the amount of extracellular matrix synthesized before its maturation. This would account for the reduced wall thickness characteristic of glucocorticoid-induced osteoporosis. The inhibition of increase in OC mRNA seen in young donor hOBs is in agreement with the known reduction in plasma OC in patients treated with glucocorticoids [Hauache et al., 1999].

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