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Inhibitory Effects of 1,25(OH)$_2$ Vitamin D$_3$ on Collagen Type I, Osteopontin, and Osteocalcin Gene Expression in Chicken Osteoblasts

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Abstract Seventeen day chicken embryonic osteoblasts treated over a 30-day period with 1,25(OH)$_2$ D$_3$ showed a 2–10-fold decrease in collagen, osteopontin and osteocalcin protein accumulation, alkaline phosphatase enzyme activity, and mineral deposition. Comparable inhibition in the steady state mRNA levels for α_1(I) and α_2(I) collagen, osteocalcin, and osteopontin were observed, and the inhibitory action of the hormone was shown to be specific for only the late release populations of cells from sequential enzyme digestions of the chick calvaria. In order to determine whether the continuous hormone treatment blocked osteoblast differentiation, the cells were acutely treated for 24 h with 1,25(OH)$_2$ D$_3$ at culture periods when the cells proliferate (day 5), a culture period when the cells cease further cell division and are increasing in the expression of their differentiated functions (day 17), and a culture period when the cells are encapsulated within a mineralized extracellular matrix (day 30). Inhibition of the expression of collagen, osteocalcin, and osteopontin were observed at days 17 and 30, while no effect could be detected for the 5-day cultures. To further define whether the inhibitory effect was specific for cells expressing their differentiated phenotype, 1,25(OH)$_2$ D$_3$ treatment was initiated at day 17 and continued to day 30 after the cells have established their collagenous matrix. In these experiments further collagenous matrix deposition, mineral deposition, alkaline phosphatase activity, and osteocalcin synthesis were also inhibited after the hormone treatment was initiated. These results, in summary, show that 1,25(OH)$_2$ D$_3$ in primary avian osteoblast cultures derived from 17-day embryonic calvaria inhibits the expression of several genes associated with differentiated osteoblast function and inhibit extracellular matrix mineral deposition. © 1995 Wiley-Liss, Inc.

Key words: chick calvaria, mineral deposition, osteopontin, osteocalcin, vitamin D$_3$

Numerous in vitro studies using bone organ cultures, a number of osteosarcoma cell lines, and primary osteoblast cell cultures have demonstrated that the steroid hormone 1,25(OH)$_2$ D$_3$ has direct effects on osteoblast function. Studies done in fetal rat calvaria organ cultures and in both primary mammalian osteoblasts or a number of different osteoblast cell lines have demonstrated decreased type I procollagen mRNA levels and protein synthesis in response to 1,25(OH)$_2$ D$_3$ [Genovese et al., 1984; Canalis and Lian, 1985; Chen et al., 1986; Franceschi et al., 1988; Rowe and Kream, 1982; Owen et al., 1991]. However, in other studies 1,25(OH)$_2$ D$_3$ has been shown to stimulate collagen type I gene expression which appeared to be dependent on differences in the differentiated states of the osteosarcoma cell lines that were been examined [Kurihara et al., 1986; Franceschi et al., 1988; Beresford et al., 1984]. The effect of 1,25(OH)$_2$ D$_3$ on the expression of noncollagenous genes more specific to the osteoblast cell lineage have focused on osteocalcin, alkaline phosphatase, osteopontin, and bone sialoprotein. Osteocalcin and osteopontin expression were shown to increase in fetal calvaria organ cultures, primary osteoblast cultures isolated from fetal rat calvaria, and in several different osteoblast cell lines treated acutely (24–48 h) with 1,25(OH)$_2$ D$_3$ [Chen et al., 1986; Lian et al., 1985; Price and Baukal, 1980; Prince and Butler, 1987; Noda and Rodan, 1989]. However, an inhibitory effect was observed for the expression of bone sialopro-

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tein [Oldberg et al., 1989]. The effects of $1,25(OH)_2$ D_3 on alkaline phosphatase expression, like type I collagen gene expression, have been more variable, with some studies showing inhibition while other studies have shown stimulation [Majeska and Rodan, 1982; Spiess et al., 1986; Manolagas et al., 1981; Franceschi and Young, 1990].

In two recent studies [Ishida et al., 1993; Owen et al., 1991] the effects of long-term hormone treatment on primary rat calvaria osteoblast cultures' processes of differentiation, extracellular matrix formation, and mineralization were examined. Both these studies demonstrated that the continuous hormone treatment over a 15–30-day period inhibited extracellular matrix mineralization and differentiated function. In the studies of Ishida et al. [1993] it was suggested that continuous hormone treatment of primary rat calvaria osteoblast cultures blocked differentiation based on dose-dependent inhibition of in vitro bone nodule formation. The study of Owen et al. [1991] examined the expression of type I collagen, osteopontin, osteocalcin, and alkaline phosphatase and demonstrated that their expression was suppressed when the cultures were treated continuously over a 30-day period with hormone. However, for short-term hormone treatment (48 h) at 6 day increments over the 30 days of culture growth of the primary cultures a stimulated expression of these genes was observed [Owen et al., 1991]. Such variability in response to the hormone might result from differential regulation by the hormone which is dependent on the osteoblast's stage of differentiation, or there may be specific populations of cells within the skeletal lineage that are differentially hormone responsive. Alternatively, the hormone may disrupt the progression of osteoblast differentiation, or alter the expression of the vitamin D_3 receptor, which then effects a given gene's response to the hormone at subsequent stages during the osteoblast's lineage progression.

Calvaria osteoblast cultures derived from 17-day embryonic chickens have been shown to have comparable aspects of maturation in vitro when compared to the mammalian systems [Gerstenfeld et al., 1987, 1988, 1990], and they exhibit a temporal sequence of gene expression similar to that observed for primary cultures of osteoblasts obtained from embryonic rat [Aronow et al., 1990], fetal bovine [Ibaraski et al., 1992], the MC3T3-E1 mouse osteoblast cell line [Franceschi and Iyer, 1992], and bone in vivo [Yoon et al., 1987; Weinreb et al., 1990]. In the present study the effects of $1,25(OH)_2$ D_3 treatment of these cultures treated continuously or acutely for 24 h at various times during their maturation in vitro with two doses of $1,25(OH)_2$ D_3 (10^{-8} 10^{-8}) were carried out to determine if the hormone had a similar response in the avian cultures as observed for the rat osteoblast cultures.

MATERIALS AND METHODS

Cell Culture

Osteoblasts were isolated by three sequential trypsin collagenase treatments of 16–17-day-old chicken calvaria [Gerstenfeld et al., 1987]. The cells released from each consecutive sequential digest are denoted as populations I, II, and III, and in all experiments described here, population III cells were used except when stated in the text. Cells were grown for 3 weeks in MEM followed by subcultivation and growth for up to 31 days in BGJ_b supplemented with 25 μg/ml ascorbate and 10 mM β-glycerophosphate. All experiments were performed using 100 mm diameter culture dishes and $1,25(OH)_2$ D_3 was added 84 h after subcultivation in concentrations ranging from 10^{-10} to 10^{-8} M by diluting a 10^{-3} M hormone stock in absolute ethanol. The endogenous level of hormone in the media containing 10% bovine serum was found to be $\sim 10^{-13}$ M from lot to lot of serum. All time points are made in reference to day 0. For continuous hormone treatments the particular hormone doses were replenished every 3 days during medium changes and no hormone addition exceeded 0.10% of the final volume of the medium with respect to the ethanol in which the hormone is suspended. For acute hormone treatments, hormone was added for 24 h with the media change on the specified days. Control cultures contained vehicle only.

Mineral and Protein Determinations

Total protein, collagen, calcium, and phosphorus accumulation were determined on samples from different time points from triplicate 100 mm dishes of at least two separate cell preparations. Samples were hydrolyzed for 24 h in 6 N HCl at 110°C in vacuo after flushing with N_2, and each sample was used for amino acid analysis on a Beckman 121M analyzer. Collagen accumulation was approximated from the hydroxy-

proline content, based on the assumption that 10% of the residues in collagen are hydroxyproline. Calcium contents were determined on the same samples by atomic absorption spectrophotometry, and phosphate content by the method of Chen et al. [1956].

Isolation and Analysis of RNA

At three time points, days 5, 17, and 30, total nucleic acid was extracted from 5–10 dishes by using a modification of the proteinase K phenol chloroform method [Gerstenfeld et al., 1984]. Total nucleic acid content per dish was determined by OD_{260} measurements and DNA was quantified by a fluorometric method [Vytasek, 1982]. RNA was separated from the total nucleic acid as previously described [Gerstenfeld et al., 1984]. Six micrograms of total RNA based on OD_{260} was loaded per lane and RNA was resolved on 1% agarose gels made 2.2 M formaldehyde. The equal loading of the RNA was verified by staining the gel with ethidium bromide before blotting onto nitrocellulose [Thomas, 1980]. ^{32}P radiolabeled cDNA probes were labeled and hybridization was carried out as described previously [Gerstenfeld et al., 1984]. Collagen pro α_1(I), pro α_2(I), pro α_1(II), pro α_1(III), chicken osteopontin, and chicken osteocalcin cDNAs were examined using cDNA clones described [Lehrach et al., 1978, 1979; Young et al., 1984; Yamada et al., 1983; Moore et al., 1991; Neugebauer et al., in press; respectively]. Relative mRNA levels were determined by scanning densitometry with an ultrascan II laser densitometer (LKB) of underexposed autoradiograms of Northern blots, and absolute mRNA levels were determined from slot blot analysis as previously described [Gerstenfeld et al., 1989], using the calculation derived from back hybridization and the RNA to DNA ratio for each RNA preparation.

Alkaline Phosphatase and Osteocalcin Determination

All assays were carried out on triplicate samples from at least two separate cells isolations. The alkaline phosphatase enzyme activity was assayed as previously described [Lowry et al., 1957]. Data is expressed as nM of p-nitrophenol released per dish/30 min/mg DNA. Medium was collected from three dishes, at either 6 or 24 h after every medium change in the presence of the hormone. Osteocalcin was then determined by RIA as previously described [Gunderg et al., 1984].

RESULTS

Effect of Long-Term $1,25(OH)_2 D_3$ on Osteoblast Growth, Extracellular Matrix Mineralization, and Cellular Differentiation

The effects of continuous $1,25(OH)_2 D_3$ treatment on cultured avian osteoblasts were examined at two hormone concentrations (10^{-8} and 10^{-10} M) over a 30-day period. Culture growth and extracellular matrix mineralization were examined by measuring total culture DNA and Ca accumulation, while the effect on osteoblast differentiated function was initially assessed by determining the culture's alkaline phosphatase enzyme activity. These results are summarized in Figure 1. No overall effect of either hormone concentration was observed for the 30-day period on the total accumulated DNA contents. However, during the first 6 days of the culture period when the cells showed their highest rate of growth, the hormone treated cultures appeared to have a slower rate of DNA accumulation, since the first time point that was examined (day 5) showed ~30% lower accumulated DNA for the hormone treated cultures (Fig. 1, upper panel). In contrast to the absence of an effect on culture growth, an 85–95% inhibition in alkaline phosphatase enzyme activity was observed for both hormone concentrations throughout the 30-day time course (Fig. 1, middle panel).

A similar inhibitory effect on the mineralization of the osteoblast culture's extracellular matrix was observed (Fig. 1, bottom panel). During the initial growth period no increase in calcium accumulation was seen for either control or hormone-treated cultures; however, after day 12 the control cultures showed their most rapid accumulation of inorganic calcium. Continuous treatment for 30 days with 10^{-8} M $1,25(OH)_2 D_3$ resulted in no increase in inorganic calcium, while the cultures treated with 10^{-10} M hormone showed a delay with increasing total inorganic calcium contents observed only after day 18. At the end point, cultures treated with 10^{-10} M in comparison to 10^{-8} M $1,25(OH)_2 D_3$ had a 1.4 vs. 9.2-fold decrease in their inorganic calcium contents, respectively. Inorganic phosphorus levels demonstrated a similar effect for both hormone concentrations as observed for inorganic calcium with a calcium to phosphorus ratio at day 30 of 2.4 for controls and 1.4 for cultures treated with 10^{-8} M $1,25(OH)_2 D_3$. The inhibition of extracellular matrix mineralization

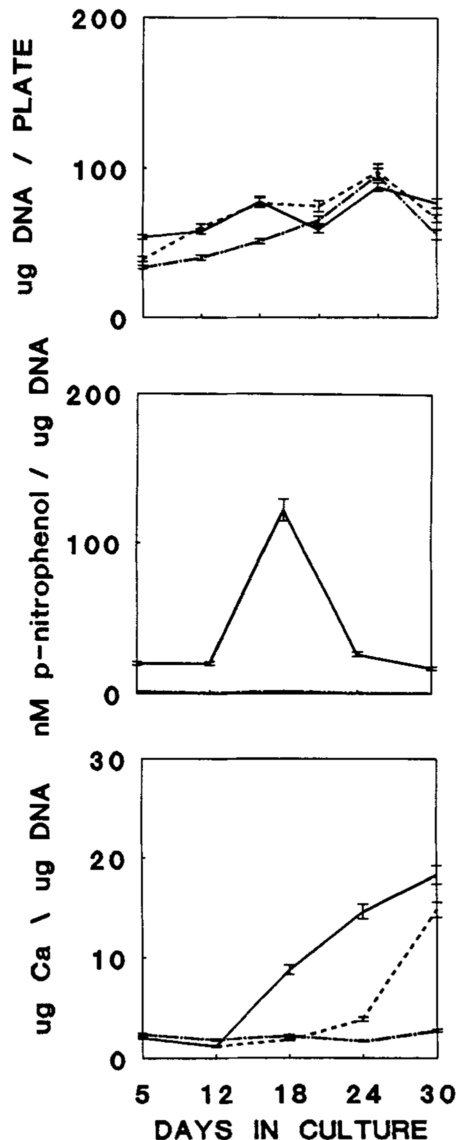

Fig. 1. Analysis of the effects of continuous $1,25(OH)_2\ D_3$ treatment on osteoblast culture growth, mineral deposition, and alkaline phosphatase enzyme activity. Triplicate samples from duplicate sets of cultures (n = 6) were collected from three experimental groups (con [-] 10^{-10}M $1,25(OH)_2\ D_3$ [---], and 10^{-8}M $1,25(OH)_2\ D_3$ [-·-·-]. All values are calculated from determinations made on samples from 100 mm dishes. Each of the determinations is denoted in the three panels. Error bars denote the standard error for each of the time points.

by $1,25(OH)_2\ D_3$ treatment was also easily observed morphologically by phase contrast microscopy, and the 10^{-8} M $1,25(OH)_2\ D_3$ treated cultures showed a marked decrease in both the number of refractile mineralized areas within the cell layer (data not shown).

The effect of continuous hormone treatment on the protein and mRNA expression of three genes known to be regulated in osteoblasts by $1,25(OH)_2\ D_3$ is presented in Figures 2, 3, and 4 for collagen, osteocalcin, and osteopontin, respectively. Three time points were examined over the 30-day treatment period that are representative of the proliferative (day 5), matrix synthetic (day 17), and mineralization (day 30) stages of the culture's maturation. Total mRNA levels were assessed by slot blot analysis and are presented graphically while the qualitative assessment of mRNA expression is shown by the Northern blot analysis.

For the analysis of osteocalcin and collagen presented in Figures 2 and 3 there was a good quantitative correspondence between the levels of protein accumulation and the level of mRNA expression for the various time points and hormone doses that were examined. These results suggest that the hormone's mode of action in affecting the accumulation of these proteins in the extracellular matrix resides primarily at a pretranslational level of regulation. Similarly, while a quantitative RIA or ELISA assay was not available for osteopontin the qualitative Western blot analysis for the 10^{-8} M hormone dose showed a similar correspondence between the mRNA expression and diminished levels of protein accumulation in the matrix. It is interesting to note that at the day 17 time point the 10^{-10} M hormone dose was slightly stimulatory for the expression both osteopontin and osteocalcin. However, the continued treatment of the cultures at this dose until day 30 had a similar dose-dependent inhibitory effect as was observed for the collagen expression.

The specificity of the hormone's effect was further confirmed by analyzing the expression of two other genes. As previously shown, type III collagen was expressed at a very low level in these cultures (~2–5% of the total collagen mRNA), and its expression remained constant throughout the culture period with neither hormone dose having an effect on its expression (data not shown). Type II collagen mRNA expression was examined to determine whether $1,25(OH)_2\ D_3$ treatment was promoting cellular differentiation into the chondroblastic lineage. At no time during these cell's growth in culture or at any hormone dose was this gene's expression detected (data not shown), further suggesting that the hormone's actions were directed toward cells within the osteoblastic lineage.

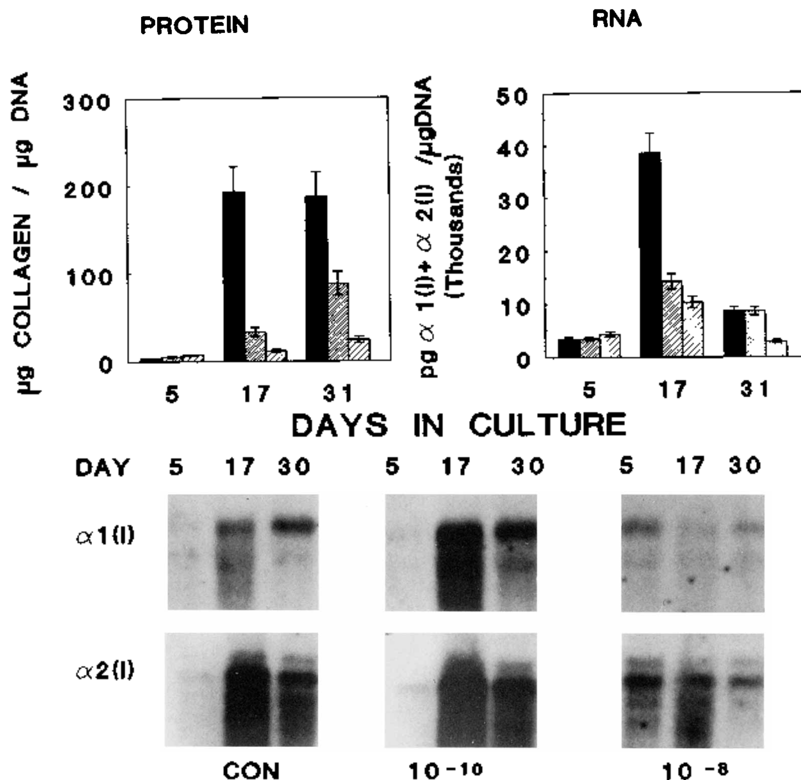

Fig. 2. Comparison of total collagen accumulation vs. total collagen type I steady state mRNA levels. Total collagen type I mRNA levels were calculated based on the addition of the quantities of the α_1(I) and α_2(I) mRNA levels that were quantified by slot blot analysis as described in the Materials and Methods. Error bars denote the total range of the error observed between two experimental determinations. Total collagen protein accumulation was calculated from triplicate samples from duplicate sets of cultures (n = 6) based on the hydroxyproline contents [con = ■ 10^{-10}M; 1,25(OH)$_2$ D$_3$ = ▨; and 1,25(OH)$_2$ D$_3$ 10^{-8}M = ▨]. Error bars of the protein determinations denote standard error for each time point. Accompanying Northern blot analyses are shown for each mRNA examined to demonstrate the specificity of each cDNA probe, the integrity of each mRNA sample, and the relative mRNA quantities. Northern blot analysis was carried out with 4 µg of total RNA per lane. The time points analyzed and experimental samples are denoted in the figure.

Specificity of 1,25(OH)$_2$ D$_3$ on Osteoblastic Cells and Effects of the Hormone Duration on Osteoblastic Response

The specificity of 1,25(OH)$_2$ D$_3$ for cells restricted to the osteoblastic lineage was shown by comparing cellular responses to the steroid for the three populations of cells released during sequential enzymatic dissociation of the 17-day embryonic calvaria (Table I). These data suggest that the cells dissociated by shorter digestion times are discrete stromal populations within bone, which express much lower levels of osteoblast-specific mRNAs and are either an earlier stage of the osteoblast lineage incapable of further development in vitro or are of some other cell lineage. It is interesting to note that these cell populations neither showed a stimulatory nor an inhibitory effect of the specific gene responses which were examined when treated with 1,25(OH)$_2$ D$_3$.

In order to determine whether the inhibitory effects of the long-term hormone treatments blocked the osteoblast's lineage progression, two different experimental approaches were carried out. In the first experimental approach the cultures were acutely treated with the hormone for 24 h at the same time points that had been examined for the continuous hormone treatment experiments. These experiments are summarized in Figure 5. These results clearly demonstrated that the acute 24 h effects of the 10^{-8} M 1,25(OH)$_2$ D$_3$ treatment were the same as observed for the continuous hormone treatment. All three genes which were examined (collagen type I, osteocalcin, and osteopontin) were comparably inhibited at each of the three time points examined, indicating that the effects of the hormone were neither restricted to a specific period during the culture's maturation nor a result of a secondary effect of a prolonged

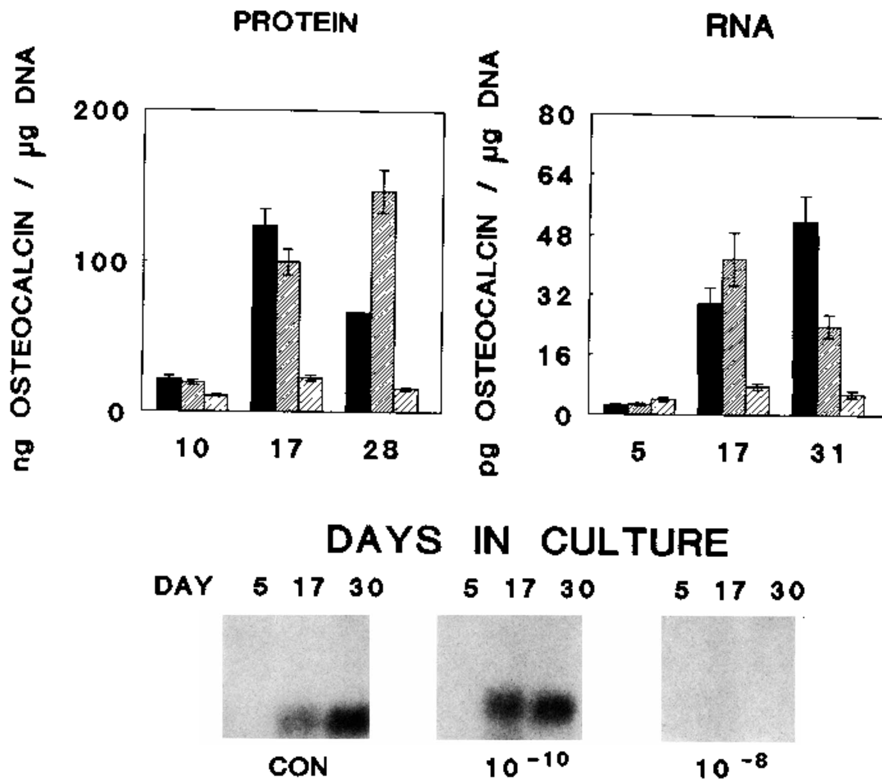

Fig. 3. Comparison of osteocalcin protein expression vs. total osteocalcin mRNA levels. Total mRNA levels were quantified by slot blot analysis as described above. Error bars denote total range of error from two separate experimental determinations. Total osteocalcin expression was calculated from triplicate samples from duplicate sets of cultures (n = 6) based on RIA analysis of the media. Experimental groups are as denoted in Figure 2. Error bars denote standard error for each time point. Accompanying Northern blot analysis are shown for each mRNA examined and were carried out on 4 µg of total RNA per lane. The time points and experimental samples are denoted in the figure.

period of the hormone treatment, blocking osteoblast differentiation.

In the second experimental approach, the effect of the hormone was examined on relatively mature cultures after 17 days of growth in cultures. These cultures are expressing high levels of osteocalcin, osteopontin, and collagen, and have accumulated an extensive collagenous matrix which has begun to mineralize. The results of these experiments are summarized in Table II. From the time at which the hormone treatment was initiated, further collagen, calcium, osteocalcin, and alkaline phosphatase enzyme activity were inhibited (Table II). In summary, the only effect of 1,25(OH)$_2$ D$_3$ treatment on primary avian osteoblasts prepared from 17-day embryonic calvaria, whether acutely or continuously exposed to the hormone and independent of the in vitro maturation state of these cultures, was inhibitory.

DISCUSSION

The initial focus of the experiments presented here was to examine the overall effect of continuous 1,25(OH)$_2$ D$_3$ on osteoblast growth and extracellular matrix accumulation in primary cultures of chicken embryonic osteoblasts. In the studies presented here the absence of any effect on total culture DNA contents by the hormone was consistent with other studies on mouse osteoblastic cell lines [Kurihara et al., 1986], while studies using primary rat osteoblasts demonstrated a decrease in proliferation [Beresford et al., 1984; Owen et al., 1991]. Biphasic effects have also been demonstrated on the proliferation of rat primary and osteosarcoma cell lines, and it has been suggested that these variable effects are dependent on the maturational state of the cells used for the study [Majeska and Rodan, 1982; Matsuma et al., 1991]. In the studies presented here, the absence of any clear

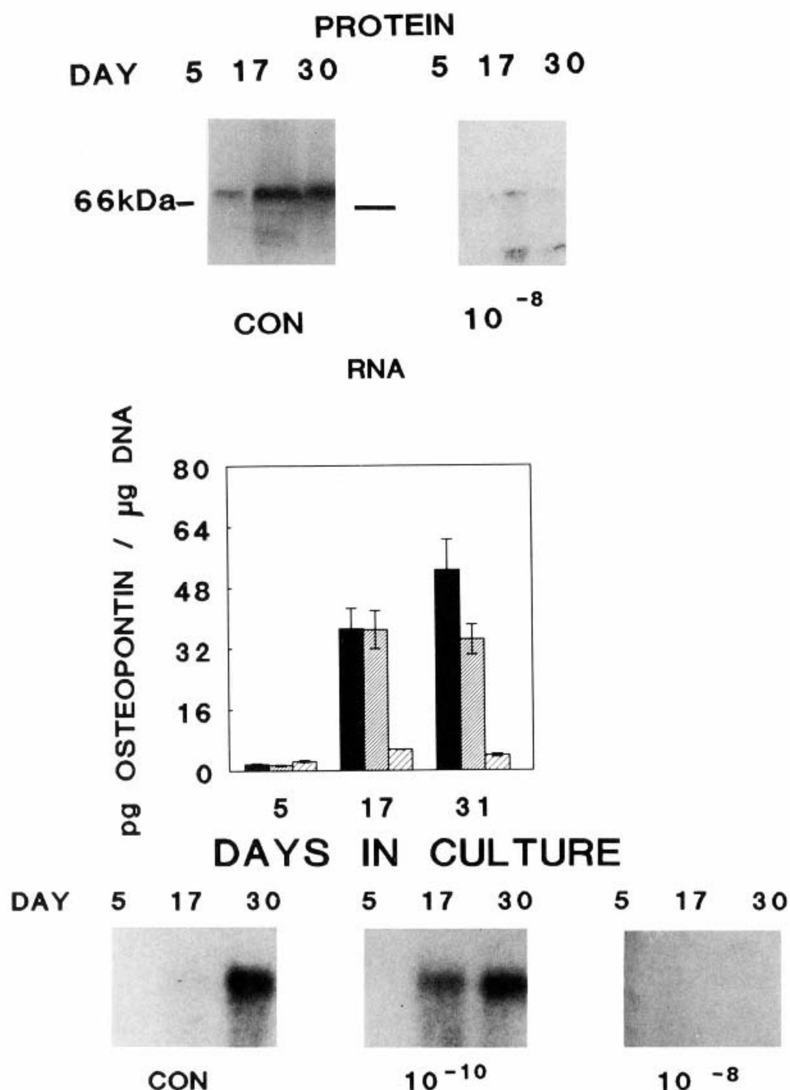

Fig. 4. Comparison of osteopontin protein accumulation vs. total osteopontin mRNA levels. Total mRNA levels were quantified by slot blot analysis as described above. Error bars denote the total range of the error from two separate experiments. Osteopontin accumulation was examined for the cell layer proteins of control and 10-8M $1,25(OH)_2$ D_3 treated cultures by Western blot analysis. Equal quantities (50 µg) of protein were electrophoresed per lane. Western blot analysis was as described in Materials and Methods. Accompanying Northern blot analysis are shown for each mRNA examined for µg of total RNA per lane. The time points and experimental samples are denoted in the figure.

effect of $1,25(OH)_2$ D_3 on cell proliferation would suggest that the phenotypic effects of the hormone on the chicken osteoblasts derived from the 17-day embryos are independent of the proliferative state of these cells.

An examination of extracellular matrix formation and total mineral accumulation showed a dose-dependent inhibition by continuous $1,25(OH)_2$ D_3 treatment. Identical results have been observed in two separate studies of primary rat osteoblast cultures [Owen et al., 1991; Ishida et al., 1993]. In vivo studies of $1,25(OH)_2$ D_3 effects on bone have shown decreases in osteoid formation and impaired mineralization in mice infused for 7 days with supraphysiological levels of $1,25(OH)_2$ D_3 [Marie et al., 1985]. Similar in vivo studies done on rats showed slightly different results in which mineralization was impaired, but an increase in osteoid was observed [Boyce and Weisbrode, 1985; Wronski et al., 1986]. Overall, both in vitro and in vivo studies done in mammals are in agreement with the observation of the studies presented here demonstrating that continuous hormone

TABLE I. Comparisons of the Effect of $1,25(OH)_2D_3$ on Osteoblast Gene Expression in Different Populations of Enzymatically Released Calvarial Osteoblasts*

	Population[a]					
	I		II		III	
	CON	D_3	CON	D_3	CON	D_3
mRNA[b]						
Type I[c]	1052	1122	1006	712	7412	1482
Osteocalcin	6.7	7.3	6.5	7.5	30	8.0
Osteopontin	1.1	1.2	1.3	1.1	37	5.5

*Populations of cells represent those cell types released by progressively longer periods of dissociation with trypsin plus collagenase from day 16–17 calvaria as previously described by Gerstenfeld et al. [1987] and outlined in the Materials and Methods section.
[a]All measurements were taken from cells grown under identical conditions, in the absence, control (CON), or presence (D_3) of 10^{-8} M $1,25(OH)_2D_3$ for 14–17 days.
[b]mRNA levels are represented as pg/ug RNA as determined from quantitative slot blot analysis. All values were determined from duplicate measurements at three concentrations. The total range of the values was $\leq 10\%$.
[c]Values are additive for both $\alpha_1(I)$ and $\alpha_2(I)$ mRNAs.

treatment inhibits mineral deposition. It is interesting to note that in both the present studies and in those of Owen et al. [1991], $1,25(OH)_2D_3$ started after the completion of collagenous extracellular matrix formation inhibited further mineralization. Such results might suggest that $1,25(OH)_2D_3$ directly affects the metabolic processes by which osteoblasts control mineralization, independent of its effects on the formation of the collagenous matrix.

Examination of the protein synthesis and gene expression of types I and III collagen, osteopontin, alkaline phosphatase, and osteocalcin was carried out for continuous hormone treatment throughout the 30-day culture period. During this time differentiated functions are increasing, osteoblasts become fully mature and are encapsulated within a mineralized matrix. The dose-dependent inhibition of collagen type I accumulation in the extracellular matrix was clearly controlled at a pretranslational level by diminished steady state mRNAs for both the $\alpha_1(I)$ and $\alpha_2(I)$ collagen genes. These results are completely consistent with other studies done with either organ cultures by Rowe and Kream [1982], and Canalis and Lian [1985], or studies done on primary rat osteoblast cell cultures by Chen et al. [1986] and Owen et al. [1991]. The maximum decrease of type I collagen mRNA levels at day 17 after acute hormone treatment can be seen at 24 h, which suggests that type I collagen gene expression is transcriptionally down-regulated by $1,25(OH)_2D_3$ in mature osteoblasts. This is also consistent with the recent data showing the presence of a conserved vitamin D response element within the 5′ promoter region of the type I collagen gene [Lichtler et al., 1989]. Type III collagen steady state mRNA levels were unaffected by either hormone dose, suggesting that the effect of the hormone was type I collagen-specific and had no effect on cells producing type III collagen (data not shown). Type III collagen mRNA represented only 2–5% of the total collagen transcript throughout the time course, providing corroborative evidence that the hormone does not selectively suppress or promote growth of undifferentiated mesenchymal or fibroblastic cells within the cultures. These results are also in agreement with other studies which have investigated the effects of $1,25(OH)_2D_3$ on type III collagen [Franceschi et al., 1988]. Type II collagen mRNA was undetectable in control and hormone-treated cultures, further indicating that the observed effects cannot be explained by the differentiation of cells to a chondroblastic phenotype (data not shown).

Alkaline phosphatase enzyme activity has been used by several laboratories as an index of an early stage of osteoblast differentiation. The effect of $1,25(OH)_2D_3$ on alkaline phosphatase enzyme activity has been more variable than that on type I collagen. Some studies have shown an increase [Manolagas et al., 1981; Ashton et al., 1985], while other studies demonstrated biphasic effects depending on the nature of the osteoblastic cell line used [Majeska and Rodan, 1982; Spiess et al., 1986] and in these studies alkaline phosphatase enzyme activity was down regulated by both continuous and acute hormone treatment. The decreased levels of osteocalcin and osteopontin expression in the continuously treated cultures prepared from 17-day chick embryos were also consistent with results obtained in primary rat osteoblast cultures [Owen et al., 1991]. In contrast, the acute treatment of the avian cultures had only an inhibitory effect on these genes expression, which is largely inconsistent with data from numerous studies in many different mammalian cell culture systems in which osteocalcin and osteopontin synthesis and mRNA levels showed marked increases after 24–48 h of $1,25(OH)_2D_3$ treatment [Prince and Butler, 1987; Yoon et al., 1988; Owen et al., 1991; Price and Baukal, 1980].

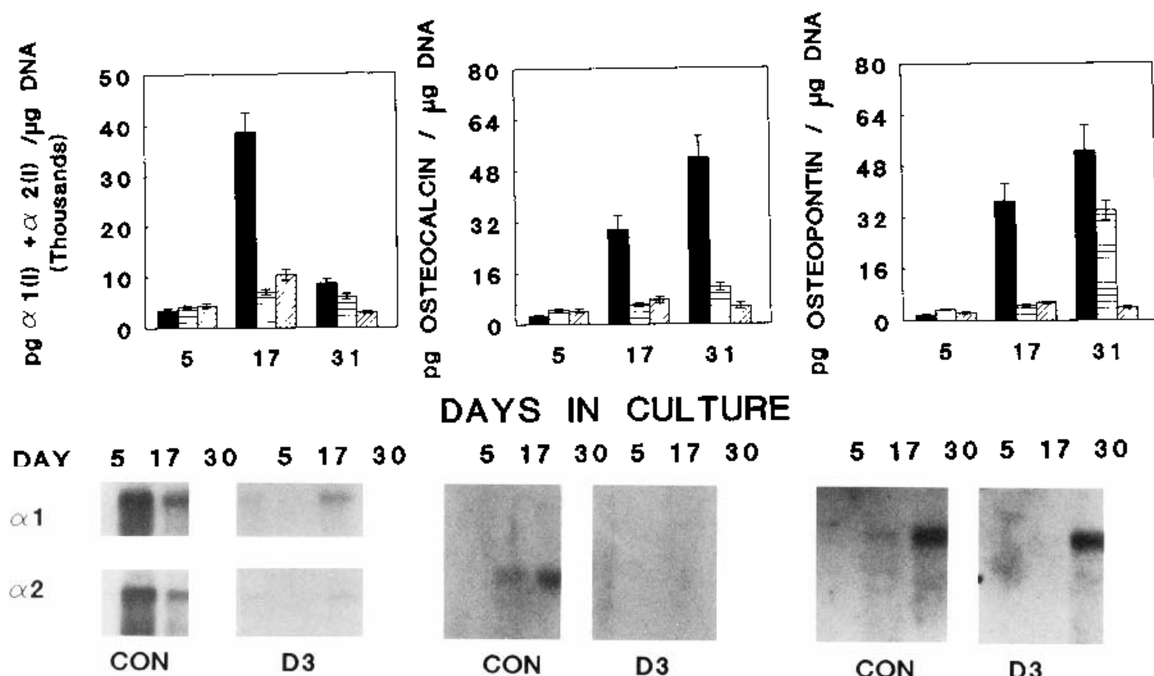

Fig. 5. Comparisons of collagen type I, osteocalcin, and osteopontin mRNA levels in cultures treated continuously vs. acutely for 24 h with 10^{-8} M $1,25(OH)_2$ D_3. Total mRNA levels were quantified by slot blot analysis as described above. Error bars denote the total range of the error from two separate experiments. Con = ■; continuous $1,25(OH)_2$ D_3 treatment at 10^{-8}M = ▨; acute $1,25(OH)_2$ D_3 at 10^{-8}M = ▤. Northern blots depict control vs. acute $1,25(OH)_2$ D_3 treated samples. Four micrograms were analyzed for each mRNA lane on the gel.

TABLE II. Effect on Continuous $1,25(OH)_2D_3$ on Cultures Treated From Day 17 to Day 30*

	Con	D_3
Collagen µg/µg DNA	165 (18.2)	105 (12.6)
Calcium µg/µg DNA	18.4 (2.4)	6.7 (12.6)
Alkaline nMpnitrophnoll phosphatase activity µg DNA	17.1 (1.9)	3.2 (0.4)
Osteocalcin ng/µg DNA	60.6 (5.4)	2.3 (0.2)

*Cultures were grown under standard conditions until day 17 at which time half the dishes were grown until day 30 in the presence of 10^{-8} M $1,25(OH)_2D_3$ supplemented with each media change. The S.D. is given for each measurement in parentheses and each experiment consisted of n ≥ 7 100 mm dishes. Measurements for collagen alkaline, phosphatase, calcium, and osteocalcin are described in the Materials and Methods.

The inconsistency in how the avian and mammalian osteoblast cultures respond to acute $1,25(OH)_2$ D_3 treatment may reside in intrinsic biological differences between birds and mammals. However, this interpretation seems unlikely since the continuous hormone treatment in avian cell cultures produces the same gene responses as seen for the mammalian cultures.

A second possible explanation of these results may exist in the different developmental stages of maturation of the calvaria used to prepare the avian and rat primary cultures. Because the 17-day avian calvaria bones are much more mineralized than the 21-day fetal rat calvaria bone, the avian cell population which has been selected for growth in culture may be more developmentally mature than the cell populations of the rat cultures. Considerable evidence exists to suggest that either the embryological developmental stage or state of an osteoblast's differentiation is of great importance in the way in which cells of the osteoblastic lineage respond to steroid hormones. In recent studies by Turksen and Aubin [1991], a sixfold difference in dexamethasome-stimulated bone nodule formation in rat embryonic calvaria osteoblast cultures was observed when alkaline and phosphatase-positive cells were immunoselected from primary cell cultures and compared to the alkaline phosphatase-depleted cultures. These authors concluded that cells at an earlier stage in the osteoblastic lineage (alkaline phosphatase-negative cells) were dexamethasome-responsive, but more mature cells (alkaline phosphatase-posi-

tive) were not. A number of reports would also suggest that the stage of cellular differentiation of different osteoblastic cell lines effect their response to $1,25(OH)_2$ D_3. In studies by Fraser et al. [1988], different rat osteosarcoma cell lines were shown to differentially respond to $1,25(OH)_2$ D_3 as exemplified by their ability to express osteocalcin or matrix gla protein. In subsequent studies, Fraser and Price [1990] demonstrated that prolonged treatment (>48 h) of ROS17.2 rat osteosarcoma cell lines with $1,25(OH)_2$ D_3 resulted in a decline in the levels of osteocalcin mRNA expression with a concomitant increase in matrix gla protein expression. These two groups interpreted the results from these studies to suggest that the regulation of the OC and MGP genes may be dependent on the developmental state of the osteosarcoma cell line and that prolonged treatment with $1,25(OH)_2$ D_3 may alter the differentiated state of a given cell line. In experiments by Franceschi et al. [1988] and Franceschi and Young [1990], these authors compared the effect of $1,25(OH)_2$ D_3 on collagen and alkaline phosphatase expression in several different osteosarcoma cell lines and proposed that $1,25(OH)_2$ D_3 may promote differentiation of mesenchymal progenitor cells but inhibit osteoblast function in more mature osteoblasts. This hypothesis is also consistent with the results reported by Owen et al. [1991] in which the greatest induction of osteocalcin and osteopontin gene expression was observed in primary rat osteoblast cultures when their basal levels of expression were the lowest. Finally, in preliminary studies comparing chicken osteoblasts prepared from 12-day vs. 17-day embryos [Gerstenfeld et al., 1992], very comparable results were obtained as those observed for the 21-day rat embryo osteoblast culture systems.

In summary, the primary effect of $1,25(OH)_2$ D_3 in cultured avian osteoblasts is to inhibit extracellular matrix protein synthesis and its mineralization. Both continuous and acute treatment of these cells inhibited the mRNA expression of collagen type I, osteopontin, and osteocalcin. Such results suggest either that the avian osteoblasts regulate these genes differently than in mammals, or that there are underlying developmental differences in the avian osteoblast populations compared to those prepared from mammalian sources which resulted in differences of the hormonal effects in the avian cell cultures.

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