Endocrine (1995) 3, 351-360

Dexamethasone promotes von kossa-positive nodule formation and increased alkaline phosphatase activity in costochondral chondrocyte cultures

Z. Schwartz[1], R.H. Hancock[2], D.D. Dean[2], B.P. Brooks[2], R. Gomez[2], A.L. Boskey[3], G. Balian[4] & B.D. Boyan[2]

[1]*Department of Periodontics, Hebrew University Hadassah Faculty of Dental Medicine, Jerusalem, Israel;* [2]*Department of Orthopaedics, The University of Texas Health Science Center, San Antonio, Texas, USA;* [3]*The Hospital for Special Surgery, New York, New York, USA;* [4]*Orthopaedic Research Laboratory, University of Virginia School of Medicine, Charlottesville, Virginia, USA*

This study examined the effect of dexamethasone on von Kossa-positive nodule formation and alkaline phosphate specific activity of costochondral chondrocytes at two distinct stages of maturation. The nodules formed by the more mature growth zone chondrocyte cultures contained von Kossa-positive deposits in the extracellular matrix that had a punctate morphology. The nodules formed by the less mature resting zone cells also contained von Kossa-positive deposits, but differentiation was delayed by three-to-five days compared to the growth zone cell cultures. Dexamethasone stimulated the number of nodules formed and shortened the length of time required for von Kossa-positive nodule formation in both types of cultures. During the first 48 h of exposure to dexamethasone, alkaline phosphatase specific activity in the cell layer of both resting zone and growth zone cultures was increased in a dose-dependent manner. At 12 days post-confluence and thereafter, enzyme activity was inhibited in the dexamethasone-treated cultures. Changes in matrix vesicle alkaline phosphatase specific activity reflected those changes seen in the cell layer after dexamethasone treatment, but with higher magnitude, suggesting that one effect of dexamethasone might be to regulate matrix vesicle function. With the exception of one culture, the chondrocytes did not synthesize type X collagen under any of the experimental conditions used. Fourier transform infrared spectroscopy (FT-IR) failed to detect the presence of calcium phosphates in any of the cultures exposed to dexamethasone except one. These results demonstrate that dexamethasone promotes early differentiation events, including nodule formation and increased alkaline phosphatase activity, in costochondral chondrocyte cultures. The failure to detect type X collagen synthesis and mineralization in both dexamamethasone-treated and control cultures suggests that these cultures lack the factors necessary for terminal differentiation and mineralization.

Keywords: steroids; dexamethasone; chondrocyte cultures; calcification; differentiation; matrix vesicles

Introduction

Corticosteroids play a major role in endochondral bone formation and directly or indirectly regulate the effects of other hormones. Their mechanism of action in bone and cartilage is not clear, and conflicting information has been reported in the literature. An excess of glucocorticoids *in vivo* results in net bone loss by causing a decrease in bone formation and an increase in bone resorption (Baylink, 1983; Burckhardt, 1984). It is also known that small amounts of glucocorticoids inhibit skeletal growth in children and animals (Loeb, 1976; Silberman, 1983; Ranz *et al.*, 1987).

The effect of glucocorticoids *in vitro* varies with the model system used. Treatment of fetal rat bone cultures with cortisol for 90 h decreases collagen production, alkaline phosphatase activity and DNA content (Dietrich *et al.*, 1979; Canalis, 1983), whereas neonatal rat bones receiving cortisol treatment for only 24 h show increases in collagen production and alkaline phosphatase activity, but decreased cell replication (Dietrich *et al.*, 1979; Canalis, 1983; Hahn *et al.*, 1984). In organ cultures of bone, dexamethasone inhibits proliferation of periosteal cells (Chyun *et al.*, 1984); however, it has also been reported that dexamethasone increases cell proliferation and nodule number and size, and promotes calcification in the presence of β-glycerophosphate (Tenenbaum & Heersche, 1982; Bellows *et al.*, 1987, 1990). In ROS 17/2.8 cell cultures, glucocorticoid treatment increases alkaline phosphatase activity and mRNA (Rodan & Rodan, 1984; Majeska *et al.*, 1985; Noda *et al.*, 1987).

In both organ and primary cell cultures and in cell lines derived from chondrosarcomas, glucocorticoids are involved in expression and maintenance of the cartilage phenotype (Jennings & Ham, 1983; Heersche *et al.*, 1984; Kato & Gospodarowicz, 1985; Takano *et al.*, 1985; Horton *et al.*, 1988; Watt, 1988; Bellows *et al.*, 1989). Dexamethasone accelerates nodule production and chondrogenesis in chick limb bud mesenchyme cultures (Biddulph *et al.*, 1988) and promotes differentiation of rat calvarial cells into chondrocytes (Bellows *et al.*, 1989; Grigoriadis *et al.*, 1988, 1989). In contrast, some studies have documented that synthesis of cartilage-specific matrix proteins is suppressed by glucocorticoids (Lebovitz & Eisenbarth, 1975; Tessler & Salmon, 1975; Silbermann, 1983; Silbermann *et al.*, 1987) and that dexamethasone inhibits cartilage growth and development, as well as production of DNA and collagen (Silbermann *et al.*, 1987; Weiss *et al.*, 1988). Chondrocytes have receptors for glucocorticoids, but the effect of hormone-binding on events such as signal transduction have not been well-characterized (Blondelon *et al.*, 1980; Silbermann & Maor, 1985; Mao & Silbermann, 1986; Hainque *et al.*, 1987; Carbone *et al.*, 1990).

Many of the effects ascribed to glucocorticoids are concentration-dependent and appear to depend on the stage of differentiation of the target cell or tissue. To examine the effects of glucocorticoids on chondrocytes at different stages of differentiation, we used an *in vitro* model developed in our laboratory (Boyan *et al.*, 1988a,b; Schwartz & Boyan, 1988; Schwartz *et al.*, 1989). The results of the present study demonstrate that chondrocyte response to dexamethasone is affected by the maturation state of the target cell, as well as dose and length of exposure to glucocorticoid. Furthermore, they suggest that dexamethasone promotes early differentiation events and stabilizes phenotypic expression at a stage of chondrogenesis prior to the fully differentiated state.

Correspondence: Barbara D. Boyan
Received 3 December 1994; accepted 2 January 1995

Results

Morphology

Both primary and fourth passage cultures exhibited comparable changes in cellular morphology with time in culture. Thus, the data presented are from fourth passage cultures, since the expansion in cell number allows for increased numbers of cells for our experiments.

At confluence, fourth passage cultures of growth zone chondrocytes were small, cuboidal, and tightly packed. During the first 11 days after achieving confluence, the morphology of the cells remained unchanged, although there were focal areas of condensation (Figure 1). Beginning at days 12–13, nodules composed of several layers of cells started to appear throughout the culture (Figure 2). By days 15–16, the nodules were increased in size, and more refractory cells were seen (Figure 3). Between days 18–24, small, punctate granular deposits were seen. These areas were von Kossa-positive and only associated with the nodules (Figures 4 and 5).

Fourth passage resting zone chondrocytes maintained in culture for up to 45 days after confluence showed changes similar to those observed for growth zone chondrocytes (data not shown). However, the morphological changes were delayed by 3 to 4 days compared with growth zone chondrocytes. Further, the appearance of von Kossa-positive deposits was delayed 5 to 6 days when compared with growth zone chondrocytes.

Similar morphologic changes were observed in cultures of both types of chondrocytes when 10^{-7} M dexamethasone was added to the medium. However, treatment with dexamethasone accelerated the observed changes by 4 to 5 days and increased both the size and von Kossa-positive staining intensity of the nodules (Figure 6). Addition of either 3 mM or 10 mM βGP to the cultures did not alter nodule formation or von Kossa staining. Addition of ATP to the culture media was toxic to the cells (Figure 7), causing the chondrocytes to become detached from the culture surface.

Mineralization

Despite the evidence of von Kossa-positive deposits in our cultures, crystalline calcium phosphates were only detected in one of the three replicate experiments. In this individual experiment, FT-IR demonstrated the presence of hydroxyapatite in growth zone chondrocyte cultures incubated for 21 days post-confluence with either 10^{-7} M dexamethasone or 10 mM βGP. Crystalline calcium phosphate was not detected in any other experiment.

Collagen production

Both growth zone and resting zone chondrocytes produced type II and type I collagen [comprised of α_1 and α_2, which migrate somewhat faster than α_1 (II)] in culture (Figure 8).

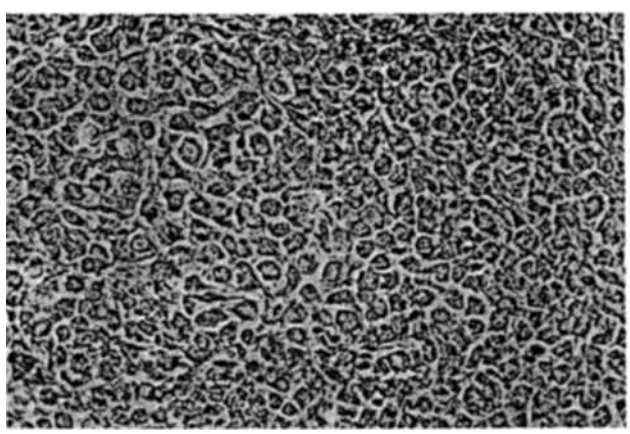

Figure 1 Reverse-phase photomicrograph of untreated fourth passage growth zone chondrocytes at 7 days post-confluence. Note the tight packing of the cuboidal cells on the surface of the plate. Magnification: 100×

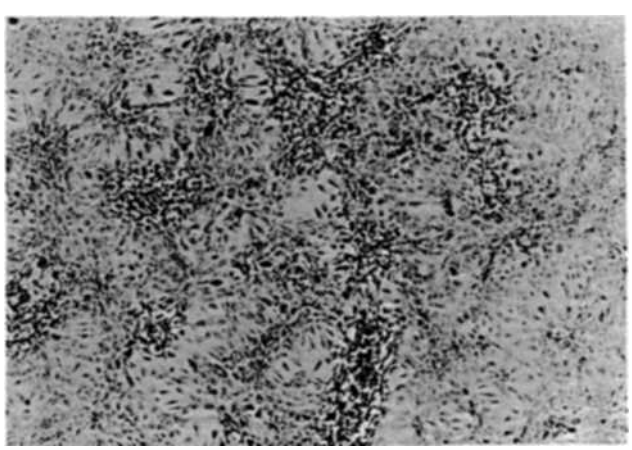

Figure 2 Reverse-phase photomicrograph of untreated fourth passage growth zone chondrocytes at 12 days post-confluence showing nodule formation. Magnification: 40×

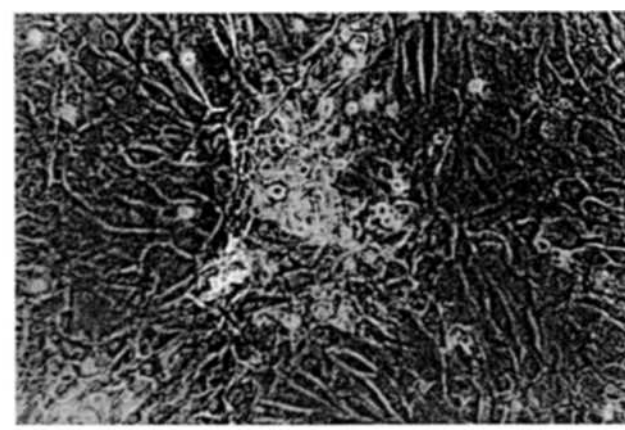

Figure 3 Reverse-phase photomicrograph of untreated fourth passage growth zone chondrocytes at 16 days post-confluence showing refractile cells associated with the nodules. Magnification: 100×

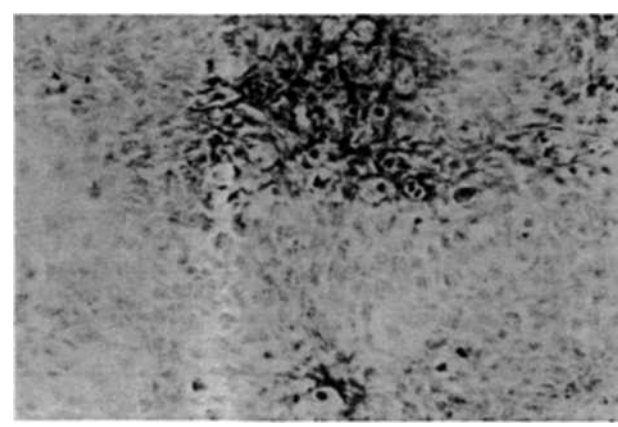

Figure 4 Light photomicrograph showing von Kossa-positive staining associated with nodules produced by fourth passage cultures of growth zone chondrocytes at 21 days post-confluence. Magnification: 100×

No type X collagen was found, even in the von Kossa-positive nodules. In only one experiment, limited pepsin digestion of proteins synthesized by confluent primary cultures of resting zone and growth zone cells produced a band at 45 kilodaltons that co-migrated with pepsin-digested pig type X collagen, but this was not a consistent observation.

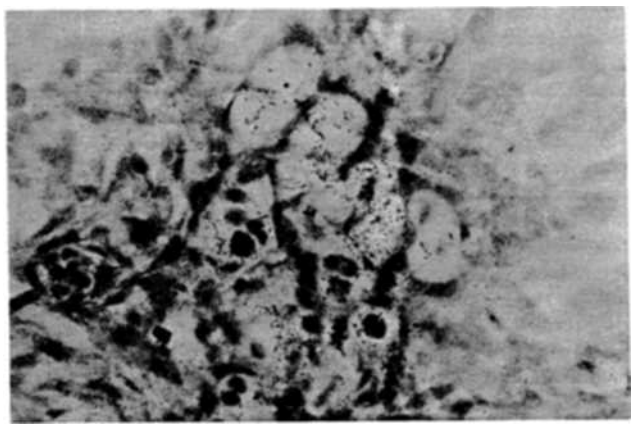

Figure 5 Light photomicrograph showing von Kossa-positive staining associated with nodules produced by fourth passage cultures of growth zone chondrocytes at 21 days post-confluence. Magnification: 200×

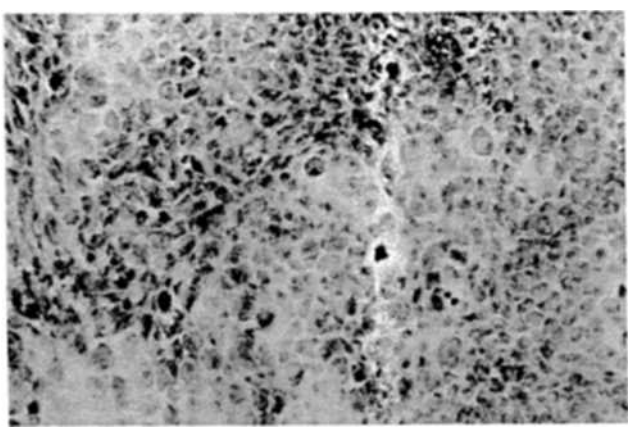

Figure 6 Light photomicrograph showing von Kossa-positive staining associated with nodules produced by fourth passage growth zone chondrocytes. Cultures were incubated with 10^{-7} M dexamethasone for 14 days post-confluence. Magnification: 100×

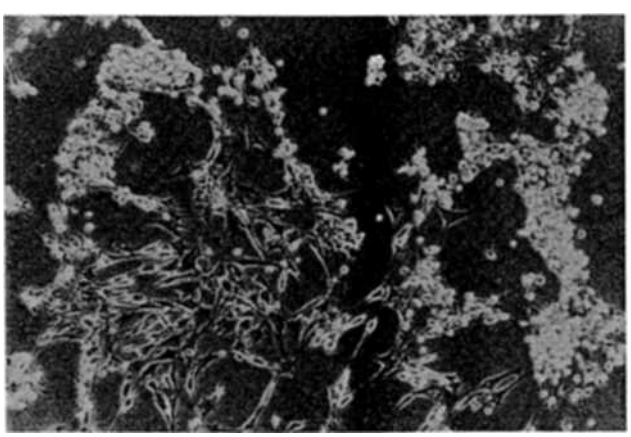

Figure 7 Reverse-phase photomicrograph of fourth passage growth zone chondrocytes at seven days post confluence. Cultures were incubated with 1 mM ATP for 5 days. Magnification: 100×

Alkaline phosphatase activity

Alkaline phosphatase specific activity in the cell layer of post-confluent growth zone and resting zone chondrocyte cultures varied as a function of time (Figure 9). In the absence of dexamethasone, no change in enzyme activity was seen in the growth zone chondrocyte cultures during the first 3 days post-confluence. At day 4, a significant increase was observed; the activity peaked at day 20, and then began to decline. By day 30, enzyme activity was still significantly higher than that observed on day 1. In contrast, resting zone chondrocyte cultures did not show a significant rise in activity until day 12, and activity peaked at day 24, with a

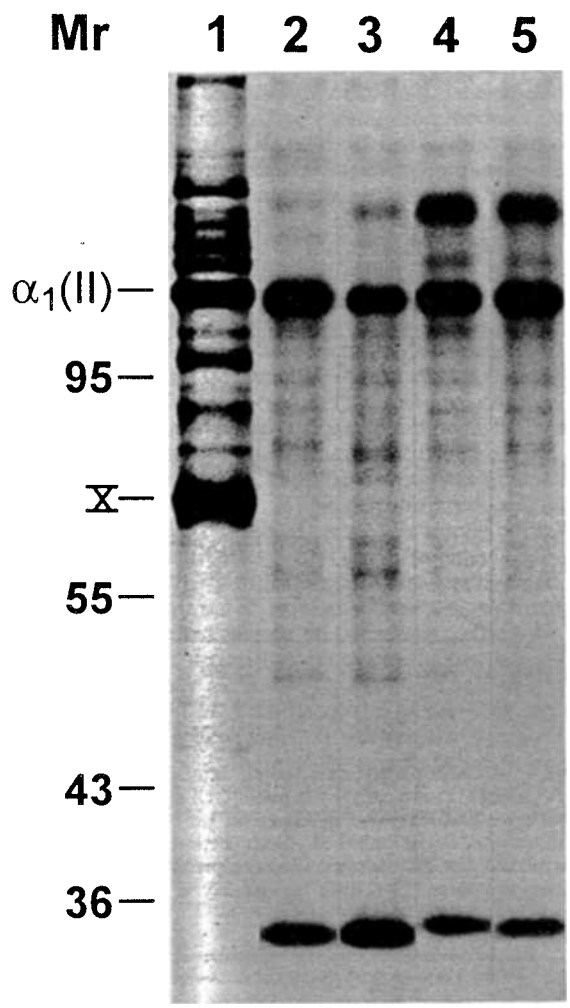

Figure 8 SDS-polyacrylamide gel electrophoresis of [^{3}H]-proline-labeled protein from cultures of fourth passage resting zone or growth zone chondrocytes. Lane 1: Proteins from cultures of chick embryo sternum chondrocytes; lane 2: Proteins from cultures of growth zone chondrocytes at 21 days post-confluence; lane 3: Proteins from cultures of growth zone chondrocytes incubated with 10^{-7} M dexamethasone at 21 days post-confluence; lane 4: Proteins from cultures of resting zone chondrocytes at 21 days post-confluence; lane 5: Proteins from cultures of resting zone chondrocytes incubated 21 days post-confluence with 10^{-7} M dexamethasone. The location of pepsin-digested type II and type X collagen on SDS polyacrylamide gels (data not shown) was confirmed with characterized type II and X collagen standards and those of globular protein markers (indicated on the left side of the fluorogram). The precursor forms of α1(I) and α2(I), which are of higher molecular weight, are above the position of the α1(II) collagen marker. Whereas all of the collagen chains were degraded by purified bacterial collagenase, the fibronectin subunit, which migrates as the uppermost band shown in the fluorogram, was unaffected by this treatment (data not shown)

value comparable to that seen on day 30 in the growth zone cell cultures.

The addition of dexamethasone to either growth zone or resting zone cell cultures for short periods of time (up to 48 h) produced dose-dependent increases in alkaline phosphatase specific activity that were significant at 10^{-8} to 10^{-6} M dexamethasone (Figure 10). The increase in enzyme activity in growth zone chondrocytes began at six hours and continued through the entire 48 h period (Figure 11). Similarly, the effect of dexamethasone on alkaline phosphatase specific activity in resting zone chondrocyte cultures was observed at 60 min and continued through the remainder of the 48 h incubation (Figure 12).

In long-term cultures incubated with dexamethasone, there was no change in alkaline phosphatase specific activity in either growth zone or resting zone cell cultures at days 3, 4, or 6 (only data from day 4 is shown in Figures 13 and 14). In growth zone chondrocyte cultures, however, there was a dose-dependent inhibition of alkaline phosphatase specific activity between 12–30 days of exposure to dexamethasone at concentrations of 10^{-7} to 10^{-6} M (Figure 13). Similar results were found when dexamethasone was added to resting zone chondrocyte cultures (Figure 14), although only 10^{-6} M dexamethasone inhibited alkaline phosphatase specific activity at days 20 and 30.

Matrix vesicles isolated from growth zone chondrocyte cultures treated with dexamethasone for up to 48 h showed a dose- and time-dependent increase in alkaline phosphatase specific activity at 10^{-7} to 10^{-6} M (Figure 15), with no significant effect observed at 10^{-9} to 10^{-8} M (data not shown). In contrast, from days 12 to 24, 10^{-7} to 10^{-6} M dexamethasone inhibited alkaline phosphatase specific activity (Figure 15). Alkaline phosphatase specific activity of plasma membrane fractions did not show any change in activity at any of the times examined or concentrations of dexamethasone used (data not shown). Matrix vesicles and plasma membranes isolated from cultures of resting zone chondrocytes exhibited responses that were similar to those observed for growth zone chondrocytes (data not shown).

Discussion

The results of this study demonstrate that chondrocytes derived from costochondral cartilage retain their ability to continue along the endochondral differentiation pathway in long-term culture. The formation of nodules containing von Kossa-positive deposits follows a sequence of maturational events similar to that seen in chondrocytes during endochondral ossification *in vivo* (Boskey, 1981; Engel *et al.*, 1994).

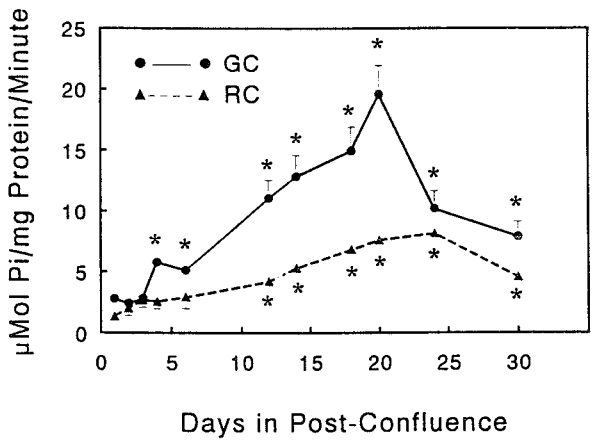

Figure 9 Alkaline phosphatase specific activity in the cell layer of fourth passage cultures of growth zone (GC) and resting zone (RC) chondrocytes. Activity was measured at the times indicated after confluence had been achieved. Each data point represents the mean ± SEM for six cultures. Each experimental condition was repeated two times. $^*P<0.05$, harvest day *vs* day 0 (confluence)

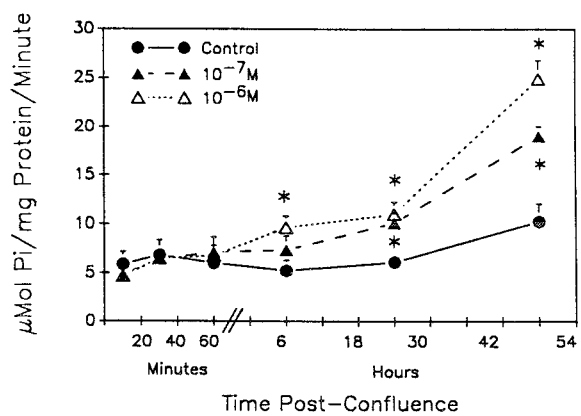

Figure 11 Time-dependent changes in alkaline phosphatase specific activity in the cell layer of fourth passage, confluent cultures of growth zone chondrocytes incubated with 10^{-7} or 10^{-6} M dexamethasone for up to 48 h. Each data point represents the mean ± SEM for six cultures. Each experimental condition was repeated two times. $^*P<0.05$, time of harvest *vs* T_0 (=confluence)

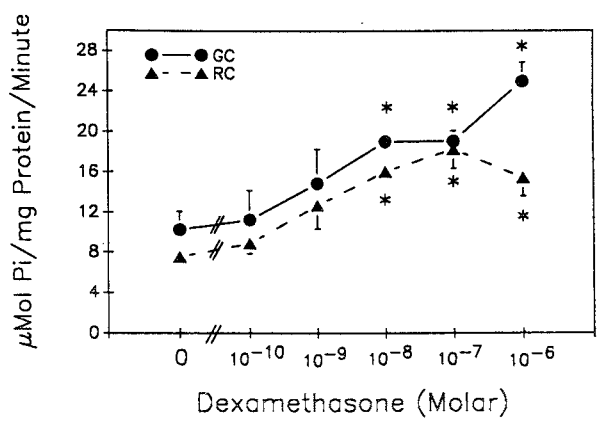

Figure 10 Alkaline phosphatase specific activity in the cell layer of fourth passage, confluent cultures of growth zone (GC) and resting zone (RC) chondrocytes incubated for 48 h with 10^{-10} to 10^{-6} M dexamethasone. Each data point represents the mean ± SEM for six cultures. Each experimental condition was repeated two times. $^*P<0.05$, treatment *vs* control

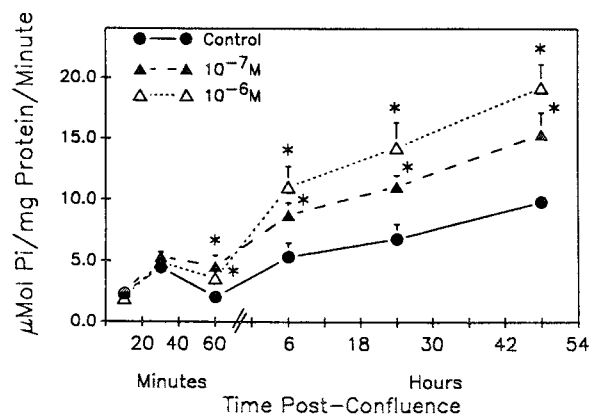

Figure 12 Time-dependent changes in alkaline phosphatase specific activity in the cell layer of fourth passage, confluent cultures of resting zone chondrocytes incubated with 10^{-7} or 10^{-6} M dexamethasone for up to 48 h. Each data point represents the mean ± SEM for six cultures. Each experimental condition was repeated two times. $^*P<0.05$, time of harvest *vs* T_0 (=confluence)

The stage of maturation of the chondrocytes *in vivo* determines the length of time needed in culture for the formation of von Kossa-positive nodules, with resting zone cells exhibiting a consistent delay compared to the more mature growth zone cells. This finding supports our earlier observation that these cells are at two distinct stages of endochondral development and that they retain their phenotypic differences through four passages in culture (Boyan *et al.*, 1988a; Schwartz & Boyan, 1988; Schwartz *et al.*, 1989).

While the chondrocytes synthesize type II collagen (Schwartz *et al.*, 1989) and immunoreactive cartilage-specific proteoglycan (unpublished data), they do not synthesize detectable amounts of type X collagen, either in monolayer or during nodule formation and differentiation, or in response to dexamethasone. In a parallel study, we examined whether the chondrocytes would synthesize type X collagen in response to two other hormones known to regulate endochondral differentiation, 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$. In neither instance was type X collagen detected (unpublished data).

The failure of these cultures to produce type X collagen at detectable levels is not due to a loss of phenotypic expression during subpassage, since primary cultures also lacked type X collagen. One possibility to account for these results is that the cells, at time of harvest from the costochondral junction, were not expressing type X collagen, and this phenotype continued subsequently in culture. In support of this idea, it is known that resting zone chondrocytes do not produce type X collagen *in vivo* and that synthesis of type X collagen in chicks, as in all species, is localized to the hypertrophic and calcifying zones of the growth plate (Schmid & Conrad, 1982; Remington *et al.*, 1984; Grant *et al.*, 1985; Kielty *et al.*, 1985; Linsenmayer *et al.*, 1991). Growth zone chondrocytes are derived primarily from upper hypertrophic cartilage, which does not produce type X collagen (Grant *et al.*, 1985), and the upper region of the lower hypertrophic cartilage. Therefore, the predominant cell type in the growth zone cultures may not have exhibited type X collagen production. In the single experiment suggesting the presence of type X in the absence of dexamethasone, it is probable that a small proportion of chondrocytes from the lower hypertrophic zone were included in the dissection of the costochondral cartilage.

The results show that type X collagen synthesis is not induced by differentiation and maturation in the culture model used, even though the cells undergo morphologic changes typical of hypertrophic, calcifying chondrocytes. In addition, synthesis of this protein is not sensitive to dexamethasone. It has been hypothesized that synthesis of this short chain collagen is specific to cartilaginous tissue on the verge of osseous transformation and that it may be involved in signaling the matrix for bone development (Silbermann, 1983). Thus, one might expect it to be produced in the long-term cultures after nodule formation had progressed, as has been reported by Coe *et al.* (1992) for chick mesenchymal cell cultures. While it is possible that factors in the culture medium suppressed type X collagen production, it is more likely that a necessary inducing agent, provided during vascularization of the calcified cartilage *in vivo*, was absent. It is also possible that some type X collagen was produced, but its synthesis was limited to focal areas. *In situ* hybridization studies will be required to verify this possibility.

Based on their ability to form von Kossa-positive nodules, the chondrocytes appeared to calcify; however, hydroxyapatite was only detected in one of three experiments we performed. Failure to detect hydroxyapatite in the majority of the experiments may have been due to the small amount of mineral present. FT-IR can detect the presence of any calcium phosphate mineral, whether amorphous or crystalline, but requires that 1% or more of the sample be in the mineral phase. Unlike bone, calcification of cartilage *in vivo* is limited and considerably less robust. Von Kossa-positive deposits in cultures of osteoblast-like cells or fetal rat calvarial cells are first observed in the nodules, but eventually spread through-

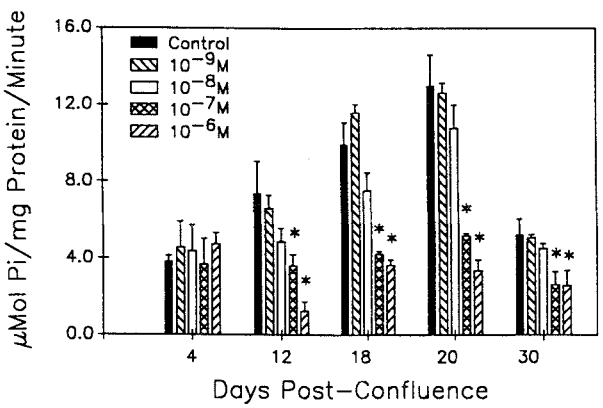

Figure 13 Dose-response and time course of alkaline phosphatase specific activity in the cell layer of post confluent, fourth passage cultures of growth zone chondrocytes incubated with dexamethasone for up to 30 days. Each data point represents the mean ± SEM for six cultures. Each experimental condition was repeated two times. $*P<0.05$, treatment *vs* control at each time point examined

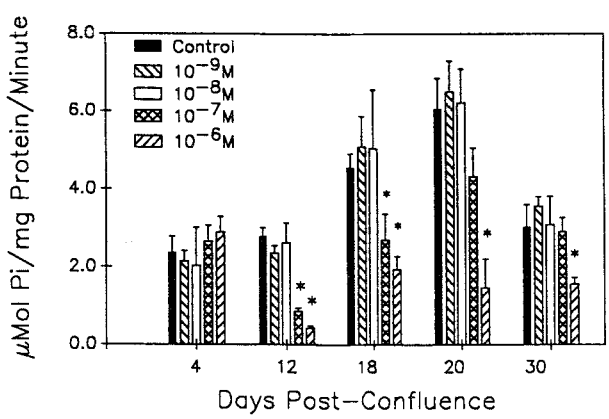

Figure 14 Dose-response and time course of alkaline phosphatase specific activity in the cell layer of post confluent, fourth passage cultures of resting zone chondrocytes incubated with dexamethasone for up to 30 days. Each data represents the mean ± SEM for six cultures. Each experimental condition was repeated two times. $*P<0.05$, treatment *vs* control at each time point examined

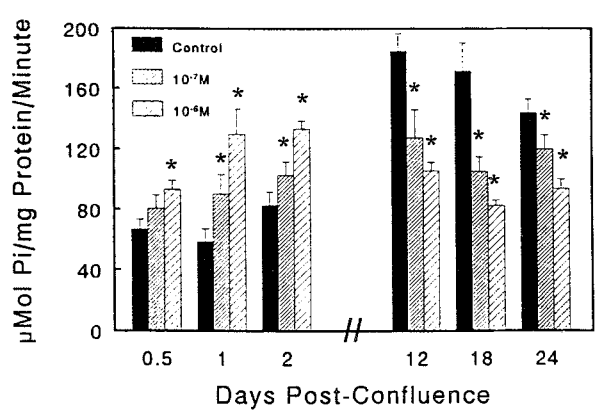

Figure 15 Effect of dexamethasone on matrix vesicle alkaline phosphatase specific activity in post confluent, fourth passage cultures of growth zone chondrocytes. Each data point represents the mean ± SEM for N = 6, where each N represents the matrix vesicles obtained from three T75 flask cultures. $*P<0.05$, treatment *vs* control at each time point examined

out the entire culture (Bellows *et al.*, 1987, 1990; Bhargava *et al.*, 1988; Ecarot-Charrier *et al.*, 1988). In the chondrocyte cultures, however, the deposits are punctate and only associated with the nodules. Taken together, it is most likely that the mineral formed by the chondrocytes in two of the three experiments was below the level of detection by FT-IR.

The homogeneity of the cells may also have limited the amount of mineral that could form. When rabbit condylar chondrocytes were cultured under comparable conditions, mineral formed within the nodule, first in association with matrix vesicles, and later within the collagenous matrix (Engel *et al.*, 1994). Normal human osteoblast-like cells (Ecarot-Charrier *et al.*, 1988) and rat osteoblast-like cells (Bhargava *et al.*, 1988) behave in a similar manner. In each of these instances, the starting population of cells was mixed with respect to maturation state. In contrast, the chondrocytes used in the present study were derived from discrete maturational zones, neither of which forms mineral *in vivo*. Even though the cells differentiate in culture, they retain phenotypic characteristics of their original maturation state.

Although matrix calcification by chondrocytes has been reported previously (Suzuki *et al.*, 1981; Vaananen *et al.*, 1983), there have been few reports showing calcification of these cells in the absence of other cell types or media supplements other than ascorbic acid (Boskey *et al.*, 1992a; Engel *et al.*, 1994). The results of the current study demonstrate clearly that normal chondrocytes will undergo chondrogenic differentiation in culture, without the addition of exogenous phosphate. Further, the ability of costochondral chondrocytes to form von Kossa-positive nodules was not markedly enhanced by β-glycerophosphate, which is commonly used to enhance the rate of calcification, or in some instances, to permit calcification (Boskey *et al.*, 1992a,b, 1994; Dean *et al.*, 1994). The data also indicate that formation of the deposits was cell-mediated and not due to dystrophic mineralization resulting from high local concentrations of phosphate.

An exception to this hypothesis may be seen in cultures incubated with ATP. ATP was shown to be an important additive for *in vitro* calcification of matrix vesicles isolated from chick chondrocytes (Chin *et al.*, 1983) and enhanced mineral deposition in chick limb culture (Boskey *et al.*, 1994). However, when ATP was added to the rat costochondral chondrocyte cultures in the present study, it appeared to be toxic to the cells. One possible explanation for the observed effect may be that dystrophic calcification was so extensive that the chondrocytes sloughed from the surface of the culture plates.

As reported for osteoblast-like cell cultures (Bellows *et al.*, 1987, 1990), dexamethasone appeared to enhance the rate of von Kossa-positive nodule formation by the chondrocytes. At early time points, dexamethasone stimulated endochondral differentiation, as evidenced by increased alkaline phosphatase activity. However, in the longer term cultures, alkaline phosphatase activity was never as high in the dexamethasone-treated cultures as it was in the untreated cells. Our data showing that dexamethasone initially stimulated and then inhibited alkaline phosphatase is similar to the findings of Canalis (1983).

By the time von Kossa-positive nodules were seen in untreated chondrocyte cultures, alkaline phosphatase specific activity was at levels seen in the dexamethasone-treated cells at earlier time points. Furthermore, dexamethasone promoted the formation of greater numbers of von Kossa-positive nodules. These observations suggest that one function of dexamethasone may be to enhance maturation toward a calcifying phenotype, but to retard terminal differentiation.

Others have shown that dexamethasone induces the expression and maintenance of the cartilage phenotype in cell and organ culture (Jennings & Ham, 1983; Heersche *et al.*, 1984; Kato & Gospodarowicz, 1985; Takano *et al.*, 1985; Horton *et al.*, 1988; Watt, 1988; Bellows *et al.*, 1989). Dexamethasone increased chondrocyte nodule number and differentiation in mesenchymal cell cultures (Biddulph *et al.*, 1988). In addition, cartilage organ cultures showed increased differentiation (Silbermann *et al.*, 1987). However, these studies failed to demonstrate von Kossa staining. Variations in experimental outcomes may be due to species differences, stage of chondrogenic maturation of the starting cell populations, or variation in culture conditions.

The present studies indicate that the effects of dexamethasone on chondrocyte differentiation are dependent, at least in part, on cell maturation. While the net effect of short-term (48 h) dexamethasone treatment on total alkaline phosphatase specific activity is comparable in resting zone and growth zone chondrocyte cultures, the time course of response is different. Resting zone chondrocytes are more sensitive to the hormone at earlier times. Stimulation of alkaline phosphatase specific activity occurs within one hour of exposure to dexamethasone in these cells, whereas stimulation of alkaline phosphatase specific activity in growth zone cells is not seen for 6 h, suggesting that dexamethasone promotes differentiation of the less mature cells. In the long-term cultures, the effects of dexamethasone occur with greater sensitivity (i.e., lower concentration) in the growth zone chondrocyte cultures after day 20, but it should be noted that enzyme activity is less in the resting zone cells and peak activity is delayed with respect to the more mature growth zone cells.

The effects of dexamethasone on alkaline phosphatase may be due to effects on matrix vesicles, which contain approximately 11% of the alkaline phosphatase in chondrocyte cell layers (Schwartz *et al.*, 1988). As was seen in the cell layer, dexamethasone stimulated matrix vesicle alkaline phosphatase specific activity at early time points, whereas, in long-term cultures, matrix vesicle enzyme activity was reduced by the hormone with respect to control. The enhanced sensitivity to dexamethasone treatment seen in matrix vesicles in comparison with changes detected in the cell layer suggests that this organelle may be a major target of dexamethasone regulation. This hypothesis is supported by the finding of Lewinson & Silbermann (1984), which showed that at concentrations of dexamethasone identical to those used in the present study, there is a marked precocious formation of matrix vesicles in growth plate organ cultures.

It is possible that the effects of dexamethasone on matrix vesicles are related to the increase in von Kossa-positive nodules. Enhanced alkaline phosphatase specific activity would lead to increased local phosphate levels detected by the stain. If local Ca concentrations remained low, hydroxyapatite formation might not occur at levels high enough to be detected by FT-IR (Boskey *et al.*, 1992b). Recent studies by Dean *et al.* (1994) have shown in osteoblast-like cells that synergistic increases in von Kossa-positive nodule formation in the presence of β-glycerophosphate and ascorbic acid are accompanied by increased matrix vesicle metalloproteinase activity. Whether dexamethasone inhibits matrix vesicle metalloproteinases is not known; however, corticosteroids have been shown to decrease proteolytic activity in articular cartilage (Martel-Pelletier *et al.*, 1985; Pelletier & Martel-Pelletier, 1989). In this situation, local phosphate levels due to matrix vesicle alkaline phosphatase would increase, but maturation of the matrix needed for bulk phase mineralization would be inhibited. Further support for the hypothesis that dexamethasone promotes initial, but not terminal, differentiation of the chondrocyte cultures is the fact that this corticosteroid inhibits phospholipase A_2 activity (Swain *et al.*, 1992), and increases in this enzyme are associated with chondrocyte maturation *in vitro* and growth plate calcification *in vivo* (Wuthier, 1973; Schwartz & Boyan, 1988). It is also possible that the maturation state of the cells *in vivo* was such that terminal differentiation of the chondrocytes to hypertrophic chondrocytes capable of calcifying their matrix could not occur under the culture conditions used. Since calcification of the costochondral cartilage in the rat is limited to a restricted region at the bone-cartilage interface, the results

may indicate the presence of a matrix constituent synthesized with or without dexamethasone that prevents calcification, even with βGP.

Materials and methods

Chondrocyte cultures

Cell isolation The chondrocyte culture model used in this study has been described in detail previously (Boyan *et al.*, 1988b). Resting zone and growth zone cartilage was dissected from the costochondral junction of 125 g Sprague-Dawley rats by sharp dissection. Care was taken to dissect out intervening tissue to limit cross-contamination of cell zones. In addition, perichondrium and calcified cartilage were discarded to limit contamination by fibroblasts and osteoblasts. Chondrocytes were isolated from the cartilage by sequential digestion with 1% trypsin (Gibco, Grand Island, NY) for 1 h and 0.02% collagenase (Worthington Type II, Freehold, NJ) for 3 h, followed by separation from the tissue debris by filtration through 40-mesh nylon and centrifugation of the filtrate at 500 *g* for ten minutes. Pelleted cells were resuspended in Dulbecco's modified Eagle's medium (DMEM), counted, and plated at an initial density of 10 000 cells/cm^2 for cells from the resting zone and at 25 000 cells/cm^2 for those from the growth zone. Cells were incubated in DMEM containing 10% fetal bovine serum (FBS), 50 μg/ml sodium ascorbate, and 1% penicillin-streptomycin-fungizone in an atmosphere of 5% CO_2 and 100% humidity at 37°C. The culture media were replaced at 24 h and then at 72 h intervals until the cells reached confluence. Cells were subcultured at confluence, using the same plating densities, and allowed to return to confluence. Either first or fourth passage cells, or both, were used for the experiments, as described below. Previous studies have shown that the cells retain their chondrogenic phenotype and differential responsiveness to vitamin D metabolites through fourth passage (Boyan *et al.*, 1988a; Schwartz & Boyan, 1988; Schwartz *et al.*, 1989).

Optimizing cell density and media composition for study of differentiation and nodule formation To determine the best cell density for nodule formation, first or fourth passage chondrocytes were incubated for 40 days in 35 mm culture dishes using one of the following protocols. (1) Resting zone cells were plated at 10 000 cells/cm^2 and growth zone cells at 25 000 cells/cm^2 in 2 ml media (Boyan *et al.*, 1988b). (2) Resting zone cells and growth zone cells were plated at 208 000 cells/cm^2 in 2 ml of media (Vaananen *et al.*, 1983). (3) Resting zone cells and growth zone cells were plated at 5×10^5 cells (1 770 000 cells/cm^2) in 0.1 ml of media in a 6 mm diameter removable cylinder placed in the center of the dish; the cylinder was removed after 24 h and 2 ml of media added (Suzuki *et al.*, 1981).

In order to optimize the formation of von Kossa-positive nodules, confluent cultures were incubated with 1 mM ATP, since this has been shown to be requried for matrix vesicle-mediated mineral deposition by chick growth plate chondrocytes *in vitro* (Chin *et al.*, 1983). Cultures were also incubated with 3 mM or 10 mM beta-glycerophosphate (βGP). In some experiments, cells were grown in modified BGJ medium (Gibco-BRL, Gaithersburg, MD), which is commonly used for mineralizing osteoblast-like cell cultures.

While the first passage chondrocyte cultures did form greater numbers of nodules, their use was impractical for many of the subsequent studies reported here. Fourth passage cells followed a comparable differentiation cascade with respect to cell and culture morphology (described in detail in Results). Seeding density did not alter nodule morphology, nor did it markedly enhance the rate or amount of von Kossa-positive nodule formation. Growth in media supplemented with βGP also resulted in no qualitative differences in von Kossa-positive nodule formation. Growth in modified BGJ medium inhibited chondrocyte proliferation and enhanced cell detachment.

Treatment of chondrocytes with dexamethasone For the reasons described above, nodule formation and differentiation were assessed using fourth passage cells seeded as described in Method no. 1 above. Cells were cultured to confluence in DMEM containing 10% FBS, 1% antibiotics, and 50 μg/ml sodium ascorbate in an atmosphere of 5% CO_2 and 100% humidity at 37°C, as described previously (Schwartz and Boyan, 1988). Culture media were changed at 72 h intervals. Dexamethasone was added to the cultures at confluence and was present in the medium throughout the remainder of the experiment. When examining the effects of dexamethasone on nodule formation and mineralization, chondrocytes were cultured for 24–30 days post-confluence. For assessment of hormone effects on alkaline phosphatase in monolayer cultures, confluent, fourth passage cells were incubated with dexamethasone for 0–48 h.

Dexamethasone was dissolved in ethanol. At the dilutions used in these experiments, the solvent does not noticeably affect the chondrocytes *in vitro* (Boyan *et al.*, 1988a). Before addition to the culture medium, the hormone stock solution (10^{-2} M) was diluted at least 1:1000 (v/v) to minimize any effect of ethanol on the cells. The medium that contained the highest concentration of dexamethasone was diluted with DMEM to prepare media with hormone concentrations ranging from 10^{-9} to 10^{-6} M. Physiologic concentrations of cortisol are 5×10^{-5} to 5×10^{-6} M. Dexamethasone, which is ten times more potent, was used at concentrations of 10^{-9} to 10^{-6} M. Therefore, the chondrocytes were exposed to physiologic concentrations of hormone or less. Each experiment included control cultures that contained ethanol at the highest concentration used as the hormone vehicle (i.e., $\leqslant 0.1\%$).

Nodule characterization

Cell morphology To resolve concerns that chondrocytes lose their differentiated phenotype with passage in culture, both first and fourth passage cells were cultured on one-chamber plastic slides (Lab-Tek Chamber Slide, Nunc, Inc., Naperville, IL). Cultures were stained for phosphate using von Kossa method. Parallel cultures were stained with toluidine blue in order to visualize nodules. Cultures were examined by reverse phase contrast microscopy.

Mineralization Von Kossa-positive deposits are often used as an indicator of calcification in culture. However, von Kossa staining only detects foci of phosphate, not necessarily calcium phosphate mineral. Therefore, attempts were made to verify that the von Kossa-positive nodules formed in culture contained hydroxyapatite. At the time of harvest, reserve zone or growth zone cells that had been cultured for 20, 24 or 30 days with or without 10^{-7} M dexamethasone, 3 mM or 10 mM βGP, or 1 mM ATP were rinsed with 50 mM ammonium bicarbonate (pH 8.0), the cell layer removed by scraping, and lyophilized. Preliminary studies indicated that the amount of mineral in these samples was insufficient to be detected by X-ray diffraction using a Debye-Scherrer powder camera with Cu Kα radiation. Subsequent samples were examined by means of Fourier transform infrared (FT-IR) spectroscopy analysis. The instrument used was a Bio-Rad FTS-40 (Bio-Rad, Cambridge, MA) coupled to a microscope attachment (UMA 100). The lyophilized cell layer was mixed with 200 mg potassium bromide (KBr) and pressed into a KBr pellet. FT-IR spectra were collected at a resolution of 4 cm^{-1}, 512 scans. To verify the results, the pellets were also examined by means of FT-IR microscopic analysis. In this configuration, the IR beam is focused through a microscope onto the sample under investigation. The area of analysis was 2 500 μm^2, and spectra were collected in the transmission mode at a resolution of 4 cm^{-1}, 1024 scans. The water vapor

contribution was subtracted, and the region of 900–1700 cm^{-1} examined. Emphasis was placed on the v_1v_3 900–1200 cm^{-1} region (mineral phosphate), as well as the 1650 cm^{-1} and 1550 cm^{-1} regions (protein amide I and II, respectively). This method will detect mineral at $\geqslant 1\%$ of the sample dry weight.

Collagen production

Type X collagen has been proposed as a marker of endochondral maturation since it is only found in the hypertrophic zone of growth plate cartilage. To determine whether resting zone or growth zone chondrocytes constitutively produce type X collagen, whether its production is related to nodule formation and calcification, and whether its production is regulated by dexamethasone, the following experiments were performed.

Metabolic labeling and protein extraction Confluent, primary cultures or fourth passage resting zone and growth zone chondrocytes were incubated for 24 h at 37°C in 10 ml DMEM containing 10% FBS, 50 μg/ml sodium ascorbate, and 50 μg/ml beta-aminoproprionitrile fumarate (BAPN) with 32 μCi/ml [^{3}H]-proline (New England Nuclear, Boston, MA). The media were collected in phosphate buffered saline (PBS) containing protease inhibitors [1 mM phenyl-methylsulfonyl fluoride (PMSF), 10 mM N-ethylmaleimide (NEM) and 25 mM ethylenediamine-tetraacetic acid (EDTA)] at 4°C. The cell layers were collected by scraping the flask mechanically and centrifuging the suspension for 15 min at 500 *g*. The cell pellet was resuspended in 5 ml PBS containing the above protease inhibitors and homogenized using a Tissumizer (Tekmar Co., Cincinnati, OH) at 4°C. The homogenate was stirred for 1 h at 4°C and centrifuged at 12 000 *g* for 30 min at 4°C. Supernatants were dialyzed against Tris-buffered saline (0.15 M NaCl, 50 mM Tris, pH 7.5) containing 0.2 mM PMSF at 4°C. The radiolabeled proteins from both media and cell layer were partially purified by precipitation with 33% saturation ammonium sulfate followed by centrifugation at 12 000 *g* for 30 min at 4°C. The pellets were resuspended in 2 ml Tris-buffered saline containing 0.2 mM PMSF.

Enzyme digestion A portion of the ammonium sulfate precipitable protein was dialyzed against 0.5 M acetic acid and limited proteolysis performed by treatment with pepsin (100 μg/ml sample) for 24 h at 4°C. Digestion was terminated by dialysis against 10 mM ammonium bicarbonate, pH 8, followed by lyophilization. After dissolving the lyophilized material at 1 mg/ml in Tris-buffered saline, some of the pepsin-digested samples underwent fractional precipitation by dialysis against 0.5 M acetic acid, pH 3.2, followed by further dialysis against consecutively increasing molarities of sodium chloride (0.9 M, 1.2 M, and 2.0 M). The precipitate at each concentration of NaCl was isolated by centrifugation, dissolved in Tris-buffered saline, dialyzed against deionized water and lyophilized.

The sensitivity of proteins to limited proteolysis was explored by treatment with pepsin under the following conditions: 2.5 mg/ml and 50 mg/ml for 60 min and 50 mg/ml for 23 h. Digestion with purified bacterial collagenase (Form III, Advanced Biofactures Corp., Lynbrook, NY) was performed by adding 50 units of collagenase in 20 μl of 10 mM calcium acetate, 25 mM Tris, pH 7.5, to 15 000 dpm of sample in 200 μl of Tris-buffered saline containing 10 mM N-ethylmaleimide and 0.2 mM phenylmethylsulfonyl fluoride and incubated for 6 h at 37°C. The reaction was terminated by freezing the sample. Collagenase-digested and control samples were prepared for gel electrophoresis by precipitation with 10% trichloroacetic acid.

Gel electrophoresis Protein extracts and pepsin digests were analysied by sodium dodecyl sulfate polyacrylamide gel electrophoresis using a 5–15% polyacrylamide gradient (Laemmli, 1970). Disulfide bonds were reduced with 50 mM dithiothreitol. Proteins were stained with Coomassie Brilliant Blue R-250, processed for fluorography with EN^{3}HANCE (New England Nuclear, Boston, MA), and exposed to presensitized X-Omat AR X-ray film (BioRad, Hercules, CA) at −70°C for seven to 10 days (Laskey and Mills, 1975).

Molecular weight estimation Molecular weights were assessed by using protein standards from Pharmacia (Piscataway, NJ). The size of collagenous proteins was estimated by comparison to collagen polypeptide (alpha) chains and to cyanogen bromide (CNBr) peptides of type I collagen (extracted from rat tail tendon) and type II collagen (from rabbit articular cartilage). CNBr peptides were prepared by chemical cleavage of purified collagen using an equal weight of CNBr in 70% formic acid for 4 h at 30°C.

Alkaline phosphatase

Alkaline phosphatase activity is associated with endochondral maturation. As chondrocytes hypertrophy *in vivo*, alkaline phosphatase specific activity increases. The increase in this enzyme is due to production of extracellular matrix vesicles, which are involved in the initiation of calcium phosphate deposition in the matrix. To determine whether the cultures exhibit this increase in enzyme activity during nodule formation, whether it is regulated by dexamethasone and whether the effect of dexamethasone at the cellular level is due to changes in matrix vesicle alkaline phosphatase specific activity, the following experiments were performed.

Preparation of cell layers/nodules Cell layers/nodules were prepared by the method of Hale *et al.* (1986). At harvest, the culture medium was removed, the flask washed two times with PBS and the cell layer detached using a cell scraper. After centrifugation, the cell layer pellet was washed twice with PBS and resuspended by vortexing in 500 μl of deionized water plus 25 μl of 1% Triton-X-100. The resulting cell layer lysate was used for biochemical analysis.

Preparation of membrane fractions Matrix vesicles and plasma membranes were prepared as previously described (Boyan *et al.*, 1988b). The conditioned medium was decanted and cells released by trypsinization (1% trypsin in HBSS). The reaction was stopped with DMEM containing 10% FBS, and the cells were collected by centrifugation at 500 *g* for 10 min, resuspended in 0.9% NaCl, washed twice, counted, and used for the preparation of a plasma membrane fraction (see below). The supernatant from the trypsin digest was centrifuged for 20 min at 13 000 *g* to sediment a mitochondria/membrane fraction. The resulting supernatant was then centrifuged for one hour at 100 000 *g* to pellet matrix vesicles.

Plasma membranes were prepared from cells isolated after trypsinization, as described above. After homogenization with a TenBroek-style homogenizer, the plasma membranes were isolated by differential centrifugation and further purified by sucrose density centrifugation (Boyan *et al.*, 1988b). Both plasma membranes and matrix vesicles were resuspended in 0.9% NaCl and stored at −70°C.

These techniques result in matrix vesicle preparations that are enriched in alkaline phosphatase specific activity that is two-to-ten times greater than that of the plasma membrane. There is a differential distribution of other plama membrane marker enzymes in the matrix vesicles (Boyan *et al.*, 1988a), as well as matrix processing enzyme (Dean *et al.*, 1992). Contamination of either membrane preparation with other subcellular organelles is minimal (Schwartz *et al.*, 1988).

Alkaline phosphatase specific activity Alkaline phosphatase [orthophosphoric monoester phosphohydrolase alkaline (EC 3.1.3.1)] activity in the cell layer, isolated plasma membranes,

and matrix vesicles was measured as a function of the release of para-nitrophenol from para-nitrophenylphosphate at pH 10.2 (Bretaudiere and Spillman, 1984). Protein was measured according to the method of Lowry *et al.* (1951).

Statistical analysis

Unless otherwise noted, the data presented in the figures and tables are from single representative experiments. For assays using cell layers, each data point represents the mean ± SEM for six cultures. For assays of matrix vesicle or plasma membrane enzyme activity, each data point represents the mean ± SEM of six samples, where each sample is the combined membranes from three-to-five cultures. Statistical significance was determined by comparing each data point to the control using the Bonferroni *t*-test ($P<0.05$). Results were validated in multiple replicate experiments. Unless otherwise stated in the Results, experiments were conducted a minimum of three times in order to verify observations.

Acknowledgements

The authors thank Sandra Messier, Rachel Quinn and Rebecca Rockey for their contributions to the preparation of the manuscript and Dr. Don Ranly for his advice. We also thank Dr. Elefteros Paschalis and Sergio Gadalette for their valuable contributions. This research was supported by PHS Grants DE-05937, DE-08603, AR-37661, and DE-04210, the VA/DoD, and the US/Israel Binational Science Foundation. Dr. Hancock was a fellow in the Air Force Institute of Technology. This study was performed in partial fulfillment of his M.S. thesis at The University of Texas Health Science Center at San Antonio. The opinions expressed in this paper do not necessarily reflect those of the US Air Force.

References

Baylink, D.J. (1983). *N. Engl. J. Med.*, **309,** 306–308.
Bellows, C.G., Aubin, J.E. & Heersche, J.N. (1987). *Endocrinology*, **121,** 1985–1992.
Bellows, C.G., Heersche, J.N. & Aubin, J.E. (1989). *Cell. Tissue Res.*, **256,** 145–151.
Bellows, C.G., Heersche, J.N. & Aubin, J.E. (1990). *Develop. Biol.*, **140,** 132–138.
Bhargava, U., Bar-lev, M., Bellows, C.G. & Aubin, J.E. (1988). *Bone*, **9,** 155–163.
Biddulph, D.M., Sawyer, L.M. & Dozier, M.M. (1988). *J. Cell. Physiol.*, **136,** 81–87.
Blondelon, D., Adolphe, M., Zizine, L. & Lechat, P. (1980). *FEBS Lett.*, **117,** 195–199.
Boskey, A.L. (1981). *Clin. Orthop. Rel. Res.*, **157,** 225–257.
Boskey, A.L., Stiner, D., Doty, S.B., Binderman, I. & Leboy, P. (1992a). *Bone Min.*, **16,** 11–36.
Boskey, A.L., Camacho, N.P., Mendelsohn, R., Doty, S.B. & Binderman, I. (1992b). *Calcif. Tissue Int.*, **51,** 443–448.
Boskey, A.L., Doty, S.B. & Binderman, I. (1994). *Microscopy Res. Tech.*, **28,** 492–504.
Boyan, B., Schwartz, Z., Carnes, D. & Ramirez, V. (1988a). *Endocrinology*, **122,** 2851–2860.
Boyan, B.D., Schwartz, Z., Swain, L.D., Carnes Jr, D.L. & Zislis, T. (1988b). *Bone*, **9,** 185–194.
Bretaudiere, J.P. & Spillman, T. (1984). *Methods of Enzymatic Analysis*, Bergmeyer, H.U. (ed.) volume 4. Verlag Chemica: Weinheim. pp. 75–92.
Burckhardt, P. (1984). *Hormone Res.*, **20,** 59–64.
Canalis, E.M. (1983). *Endocrinology*, **12,** 931–939.
Carbone, J.P., Badridge, R.C., Koszalka, T.R., Bongiovanni, A.M. & Brent, R.L. (1990). *J. Steroid Biochem.*, **35,** 495–505.
Chin, J.E., Hale, J.E., Ishikawa, Y., Register, T.C. & Wuthier, R.E. (1983). *Int. Conf. on Calcium Regulating Hormones.* Elsevier Science Publishers B.V.: Amsterdam. pp. 454–458.
Chyun, Y.S., Kream, B.E. & Raisz, L.G. (1984). *Endocrinology*, **114,** 477–480.
Coe, M.R., Summers, T.A., Parsons, S.J., Boskey, A.L. & Balian, G. (1992). *Bone Min.*, **18,** 91–106.
Dean, D.D., Schwartz, Z., Muniz, O.E., Gomez, R., Swain, L.D., Howell, D.S. & Boyan, B.D. (1992). *Calcif. Tissue Int.*, **50,** 342–349.
Dean, D.D., Schwartz, Z., Bonewald, L.F., Muniz, O.E., Morales, S., Gomez, R., Brooks, B.P., Qiao, M., Howell, D.S. & Boyan, B.D. (1994). *Calcif. Tissue Int.*, **54,** 399–408.
Dietrich, J.W., Canalis, E.M., Maina, D.M. & Raisz, L.G. (1979). *Endocrinology*, **104,** 715–721.
Ecarot-Charrier, B., Shepard, N., Charette, G., Grynpas, M. & Glorieux, F.H. (1988). *Bone*, **9,** 147–154.
Engel, F.E., Swain, L.D., Dean, D.D. & Boyan, B.D. (1994). *Acta Anat.*, **150,** 136–149.
Grant, W.T., Sussman, M.D. & Balian, G. (1985). *J. Biol. Chem.*, **260,** 2798–3803.
Grigoriadis, A.E., Heersche, J.N.M. & Aubin, J.E. (1988). *J. Cell. Biol.*, **106,** 2139–2151.
Grigoriadis, A.E., Aubin, J.E. & Heersche, J.N. (1989). *Endocrinology*, **125,** 2103–2110.
Hahn, T.J., Westbrook, S.L. & Halstead, L.R. (1984). *Endocrinology*, **114,** 1864–1870.
Hainque, B., Dominice, J., Jaffry, P., Ronot, X. & Adolphe, M. (1987). *Ann. Rheum. Dis.*, **46,** 146–152.
Hale, L.V., Kemick, M.L. & Wuthier, R.E. (1986). *J. Bone Min. Res.*, **1,** 489–495.
Heersche, J.N.M., Pitaru, S., Aubin, J.E. & Tenenbaum, H.C. (1984). *Endocrine Control of Bone and Calcium Metabolism.* Cohn, D.V., Fujita, T., Potts, J.T. & Talmage, R.V. (eds) volume 8A. Elsevier: Amsterdam. pp. 147–150.
Horton Jr, W.E., Cleveland, J., Rapp, U., Nemuth, G., Bolander, M., Doege, K. & Yamada, Y., Hassell, J.R. (1988). *Exp. Cell. Res.*, **178,** 457–468.
Jennings, S.D. & Ham, R.G. (1983). *Cell. Bio. Int. Rep.*, **7,** 149–159.
Kato, Y. & Gospodarowicz, D. (1985). *J. Biol. Chem.*, **260,** 2364–2373.
Kielty, C.M., Kwan, A.P.L., Holmes, D.F., Schor, S.L. & Grant, M.E. (1985). *Biochem. J.*, **227,** 545–554.
Laemmli, U.K. (1970). *Nature*, **227,** 680–685.
Laskey, R.A. & Mills, A.D. (1975). *Eur. J. Biochem.*, **56,** 335–341.
Lebovitz, H.E. & Eisenbarth, G.S. (1975). *Vit. Horm.*, **33,** 575–648.
Lewinson, D. & Silbermann, M. (1984). *Calcif. Tissue Int.*, **36,** 702–710.
Linsenmayer, T.F., Chen, Q., Gizney, E., Gordon, M.K., Marchant, J.K., Mayne, R. & Schmid, T.M. (1991). *Development*, **111,** 191–196.
Loeb, J.N. (1976). *N. Engl. J. Med.*, **295,** 547–552.
Lowry, O.H., Rosebrough, N.J., Farr, A.L. & Randall, R.J. (1951). *J. Biol. Chem.*, **193,** 265–275.
Majeska, R.J., Nair, B.C. & Rodan, G.A. (1985). *Endocrinology*, **116,** 170–179.
Mao, G. & Silbermann, M. (1986). *J. Craniofac. Genet. Dev. Biol.*, **6,** 189–202.
Martel-Pelletier, J., Cloutier, J.-M., Howell, D.S. & Pelletier, J.-P. (1985). *Arth. Rheum.*, **28,** 405–412.
Noda, M., Yoon, K., Thiede, M., Buenaga, R., Weiss, M., Henthorn, P., Harris, H. & Rodan, G.A. (1987). *J. Bone Min. Res.*, **2,** 161–164.
Pelletier, J.-P. & Martel-Pelletier, J. (1989). *Arth. Rheum.*, **32,** 181–193.
Ranz, F.B., Aceitero, J. & Gaytan, F. (1987). *J. Anat.*, **154,** 73–79.
Remington, M.C., Bashey, R.I., Brighton, C.T. & Jimenez, S.A. (1984). *Biochem. J.*, **224,** 227–233.
Rodan, G.A. & Rodan, S.B. (1984). *Bone and Mineral Research Annual.* Peck, W.A. (ed.) volume 2. Elsevier: Amsterdam, pp. 244–285.
Schmid, T.M. & Conrad, H.E. (1982). *J. Biol. Chem.*, **257,** 12451–12457.
Schwartz, Z. & Boyan, B. (1988). *Endocrinology*, **122,** 2191–2198.
Schwartz, Z., Knight, G., Swain, L.D. & Boyan, B.D. (1988). *J. Biol. Chem.*, **263,** 6023–6026.
Schwartz, Z., Schlader, D., Ramirez, V., Kennedy, M. & Boyan, B. (1989). *J. Bone Mineral Res.*, **4,** 199–207.
Silbermann, M. (1983). *Cartilage.* Hall, B.K. (ed.) volume 2. Academic Press: New York. pp. 327–368.
Silbermann, M. & Maor, G. (1985). *Acta Endocrinol.*, **108,** 343–350.

Silbermann, M., von der Mark, K., Maor, G. & van Menxel, M. (1987). *Bone Mineral.*, **2,** 87–106.
Suzuki, F., Takase, T., Takigawa, M., Uchida, A. & Shimomura, Y. (1981). *Proc. Natl. Acad. Sci. USA*, **78,** 2368–2372.
Swain, L.D., Schwartz, Z. & Boyan, B.D. (1992). *Biocheim. Biophys. Acta.*, **1136,** 45–51.
Takano, T., Takigawa, M. & Suzuki, F. (1985). *J. Biochem.*, **97,** 1093–1100.
Tenenbaum, H.C. & Heersche, J.N. (1982). *Calcif. Tissue Int.*, **34,** 76–79.
Tessler, R.H. & Salmon Jr, W.D. (1975). *Endocrinology*, **96,** 898–902.
Vaananen, H.K., Morris, D.C. & Anderson, H.C. (1983). *Metab. Bone Dis. Rel. Res.*, **5,** 87–92.
Watt, F.M. (1988). *J. Cell. Sci.*, **89,** 373–378.
Weiss, A., Livne, E. & Silbermann, M. (1988). *Growth Dev. Aging*, **52,** 67–75.
Wuthier, R.E. (1973). *Clin. Orthop. Rel. Res.*, **90,** 191–200.