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EFFECT OF DEXAMETHASONE ON CHONDROCYTE CULTURES

A

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San Antonio, Texas

May, 1990

EFFECT OF DEXAMETHASONE ON CHONDROCYTE CULTURES

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EFFECT OF DEXAMETHASONE ON CHONDROCYTES IN CULTURE

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During bone induction by demineralized bone the most robust bone formation is associated with calcified cartilage. In order to examine this important component of the bone induction cascade, we examined whether chondrocytes could mineralize their matrix in culture. Cultures were treated with dexamethasone to determine whether this drug would promote chondrogenesis and

calcification in a manner comparable to that seen in osteoblasts.

In vitro studies have shown that dexamethasone has numerous effects on cell differentiation and maturation. When osteoblasts isolated from fetal rat calvaria are incubated with dexamethasone, they form multilayer nodules which support mineral deposition in vitro. Similar observations have been made using normal human osteoblasts. If dexamethasone is not supplied to the cells, nodule formation and mineralization either fail to occur or are greatly reduced, indicating that it stimulates cell differentiation.

Chondrocytes were obtained from the resting or growth zone cartilage from the costochondral junction of 125g male Sprague Dawley rats. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), vitamin C and antibiotics in an atmosphere of 5% CO_2 in air at 37°C. At confluence, cells were allowed to form multilayer nodules for an additional 30 days. One-half the cultures received 10^{-5} to 10^{-10}M dexamethasone. Cultures were examined daily using inverse phase microscopy and photographed. In some experiments, cultures were stained with toluidine blue for proteoglycans and with von Kossa stain for deposition of mineral. Alkaline phosphatase specific activity was determined as a function of the release of para-nitrophenol from para-nitrophenyl phosphate.

Primary cultures produced nodules and calcified in culture without dexamethasone; however, both nodule formation and calcification occurred earlier and in greater quantity with dexamethasone treatment. There was a significant increase in alkaline phosphatase specific activity in growth zone cells after 24 and 48 hours with 10^{-6} and 10^{-7}M dexamethasone. Enzyme activity in the control cultures increased at day 6 to levels comparable to the treated cultures. After day 6, alkaline phosphatase in control cultures continued to increase, peaking at day 24 and returning to levels comparable to the dexamethasone cultures by day 30. Resting zone cells followed a similar pattern but exhibited a 7-day delay.

These data suggest that dexamethasone may play a role by mediating alkaline phosphatase specific activity and nodule formation. Dexamethasone may stimulate cell proliferation and differentiation and rapidly elevate enzyme activity to levels optimal for calcification. In non-dexamethasone-treated cultures, increased enzyme activity may occur later but at higher levels. However, calcification may not occur until alkaline phosphatase activity is decreased to optimal levels. These data suggest that dexamethasone may be useful for promoting endochondral bone repair.

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I. INTRODUCTION

A. Endochondral bone formation.

Bone formation within a cartilage matrix is the primary method of skeletal formation in vertebrates. In humans, only the flat bones of the skull, the irregular bones of the face, and the clavicle are formed from purely intramembranous bone formation (Hall, 1988). Bone wounds heal by a process that is similar to the endochondral ossification which occurs during normal bone growth and development. This process can be induced by demineralized bone and various bone-derived factors when they are implanted in mesenchymal tissues (Urist et al., 1983). Investigators have proposed that demineralized bone, and the factors which can be isolated from it, can be used to promote bone repair in tissues that would otherwise not form bone. Nilsson et al. (1986) have used bone morphogenetic protein (BMP), a factor present in bone, to treat large segmental bony defects in dog ulnas and Schmitz (1987) developed copolymers containing bone inductive material which promoted healing in non-union cranial defects.

Normal endochondral bone formation and bone repair are similar but have some important differences. Bone repair is influenced by intrinsic and extrinsic bioactive factors such as BMP and platelet derived growth factor (PDGF) respectively. Some factors may not even be present during embryologic bone development. A 31,000 dalton protein referred to as chondrocyte

stimulating activity (CSA) which stimulates the conversion of mesenchymal cells into chondrocytes is not present in chick embryo limbs until after there has been significant limb development (Kujawa et al. 1986, Peachak et al. 1986, Caplan 1986).

The mineral phase of bone and cartilage is a poorly crystalline form of hydroxyapatite. Initial calcification involves the formation of a critical nucleus which is the smallest stable combination of ions with the structure of the crystalline material that can persist in solution. After this nucleation, additional ions are added and crystal growth can proceed. Mineralization of bone during normal development depends on an interaction of cells, extracellular macromolecules, and matrix components (Boskey, 1981).

The cell appears to the primary factor in the regulation of mineralization through enzymes such as alkaline phosphatase and inorganic pyrophosphatase. Cell storage of calcium and phosphate can create a microenvironment in which calcification can occur by release of calcium and phosphates by mitochondria in the presence of vitamin D (Brighton and Hunt, 1978). In cartilage and primary bone formation, the initial deposition of hydroxyapatite occurs in association with extracellular matrix vesicles (Anderson, 1969; Bonucci, 1970; Sela et al., 1978). Calcification of matrix vesicles proceeds by the accumulation of calcium which occurs because the membrane acidic phospholipids

have a high affinity for this ion (Boskey, 1978; Wuthier and Gore, 1977). Once the initial calcium complexes have formed on the vesicle membrane, apatite proliferation proceeds until the vesicle breaks up from the presence of the crystals. Extracellular macromolecules such as collagen and noncollagenous proteins may regulate calcification by providing epitaxial sites for apatite growth, serving as hydroxyapatite nucleators, stabilizing apatite precursors, and regulating the size and orientation of the deposited mineral (Boskey, 1981). Collagen provides an oriented support for newly formed crystals and may regulate mineral size in conjunction with other proteins (Boyde et al., 1978).

B. **In-vitro models of calcification.**

1. Osteoblast culture models. Osteoblasts isolated from the calvaria of 5-6 day old mice have demonstrated the ability to calcify their matrix in culture when B-glycerolphosphate is added (Ecarot-Charrier et al., 1983). In that model, the calvarial sections are placed in tissue culture media with glass coverslip fragments on the endocranial surface. After 4 days the cells which have migrated onto the glass fragments are scraped and grown in the culture media. Similarly, osteoblasts isolated from 21 day fetal rat calvaria have also been shown to mineralize their matrix in culture (Bellows et al., 1986). In the fetal rat calvarial model, the osteoblasts are isolated by

enzymatically releasing the cells by sequential digestion with collagenase. In this model, the addition of organic phosphate was necessary for mineralization in the cultures.

2. Chondrocyte calcification in-vitro. Calcification of the cartilage matrix remains a hallmark of endochondral bone formation whether due to normal bone growth, fracture repair or bone induction. The details of the behavior of chondrocytes during chondrogenesis along a calcified cartilage pathway are only now being clarified. There is microscopic and biochemical evidence that epiphyseal chondrocytes produce extracellular matrix vesicles as they differentiate from the proliferative to hypertrophic cell zones (Anderson, 1969). These matrix vesicles become the first site of crystal deposition after cell hypertrophy (Brighton and Hunt, 1976).

Evidence of chondrocyte calcification in vitro has only recently been demonstrated using rabbit chondrocytes in a culture that involved pelleting cells into a centrifuge tube (Kato et al., 1988). Prior to 1988, chondrocyte calcification in vitro was reported by Vaananen and co-workers (1983). Their study used rachitic rat epiphyseal growth cartilage which demonstrated matrix vesicle calcification but gave little insight into calcification in normal chondrocytes. Chondrocytes from rat costal cartilage demonstrated matrix vesicle-associated calcification when co-cultured with bone marrow cells, or non-

specific calcification in a system which contained a phosphate level which had been artificially elevated (Suzuki et al., 1981).

The details of the activity of calcifying chondrocytes as they differentiate are only now being elucidated. A new model of the normal chondrocyte in culture was established by Boyan and co-workers (1988). They demonstrated that chondrocytes derived from the resting and growth zones of costochondral cartilage maintain differential phenotypic expression in culture. The cells produce different extracellular matrix (Schwartz et al., 1988). The matrix vesicles produced by cells from the two zones differ in enzyme activity (Boyan et al., 1988). The matrix vesicles produced by resting zone cells contain less alkaline phosphatase activity than do the matrix vesicles produced by growth zone cells. In both cell types, the specific activity of alkaline phosphatase in the matrix vesicle fraction is greater than seen in the cell membranes. 1,25-$(OH)_2$ D_3 stimulates primarily growth zone cell matrix vesicle enzymes, but does not affect these enzymes in matrix vesicles produced by resting zone cell cultures (Boyan et al., 1988; Schwartz et al., 1988). 24,25-$(OH)_2D_3$ stimulates primarily the matrix vesicle enzymes in resting zone cell cultures, but not in the growth zone cell cultures. These studies have established that chondrocytes from different zones of chondrogenic differentiation can be cultured, that they can maintain their

phenotypic expression, that their matrix vesicles have distinct biochemical characteristics, and that at different stages of differentiation, chondrocytes respond differently to Vitamin D metabolites.

C. **The effect of dexamethasone on calcification.**

1. Clinical effects of dexamethasone. Dexamethasone is a 392.47 molecular weight synthetic adrenocortical steroid approximately 33 times more potent than Cortisone. Dexamethasone is widely used in surgical settings which require a minimum post-operative inflammatory response. Patients undergoing lumbar laminotomy or discectomy, when given dexamethasone intra and post-operatively, experienced less pain and used significantly less narcotic (King, 1984). Dexamethasone has been used in the management of cancer patients with odontoid fractures (Sudaresan et al., 1981).

The use of steroids is not without its problems. It is widely accepted that long-term high-dose steroid therapy previously used in renal transplants was noted to cause avascular bone necrosis, and short-term high-dose steroid therapy has been reported to cause multifocal avascular necrosis (Taylor, 1984).

2. In-vivo effects. Experimental studies have demonstrated that rats treated prenatally with dexamethasone have inhibited

differentiation and growth of epiphyseal cartilage (Dearden et al., 1986). Weiss and co-workers (1986) demonstrated that dexamethasone has an inhibitory effect on proteoglycan synthesis and a depressive effect upon the degradation of proteoglycans, and thereby interferes with the normal growth and development of condylar cartilage in newborn mice. In Wistar rats, with increasing doses of dexamethasone there is a decrease in the amount of rough endoplasmic reticulum and Golgi apparatus in chondrocytes from the knee (Podbielski and Raiss, 1986). In the same study, degenerative changes were manifested in lipid droplets and myeloid bodies which exhibited a dose dependent increase. Luo and Murphy (1989) demonstrated that dexamethasone will significantly inhibit growth hormone induced production of insulin-like growth factor mRNA. The cartilage of pony foals treated with dexamethasone long term demonstrated degenerative lesions in the cartilage and bone of the proximal femur (Glade et al., 1983).

3. In-vitro effects of dexamethasone. The effect of dexamethasone in-vitro varies widely depending on the cell type, stage of differentiation, dosage of steroid, and the time of steroid administration. In a bone-derived clonal mesenchymal cell population, four distinct morphologic phenotypes differentiated when cultured with dexamethasone; muscle cells, adipose cells, chondrocytes, and bone-forming cells (Grigoriadis et al., 1988). Chondrocyte nodules appeared after 16 days and

mineralized bone nodules were observed after 21 days. In a series of articles, Bellows and co-workers (Bellows et al., 1986; Bellows et al., 1987; Bellows et al., 1989; Bellows and Aubin, 1989) have described the effects of dexamethasone on cultured cells isolated from rat calvaria. Dexamethasone caused a dose-related increase in discrete three-dimensional mineralization nodules which peaked at 10^{-8} M compared to controls. Higher concentrations of dexamethasone did not increase the number of nodules indicating that constant exposure to low levels of steroid were most beneficial at inducing mineralization (Bellows et al., 1987). In dexamethasone supplemented cultures of cells isolated from fetal rat calvaria, two types of nodules were observed. Cartilaginous nodules contained type II collagen and bone nodules contained type I collagen (Bellows et al., 1989). The nodules were distinct and developed in isolation from each other. The fetal rat calvarium has a distinct patch of cartilage and the authors believe that the cartilaginous cells probably came from that area. The bone nodules continued to increase to day 28 but the cartilaginous nodules did not increase after the cells reached confluency. Using the same model, Bellows and Aubin (1989) demonstrated that the use of dexamethasone increases the bone-forming cell expression and demonstrated that one osteoprogenitor cell gives rise to one bone nodule.

Cultured osteoblast-like rat bone cells have shown a variable proliferative response to dexamethasone depending on the cell density at the time of exposure (Chen et al., 1983). In extremely low density cultures dexamethasone had an inhibitory effect, whereas it had a stimulatory effect in dense cultures. Osteoblast-like rat bone cells have been demonstrated to possess a specific receptor for insulin-like growth factor I; dexamethasone increased the binding at specific time periods (Bennett et al., 1984) and slightly increased osteocalcin synthesis at low doses (Chen et al., 1986). Dexamethasone has shown a short-term stimulatory effect on alkaline phosphatase activity in cells from the rat calvaria, which is followed by an inhibitory effect after long term exposure, which was related to a generalized decrease in cell population (Canalis, 1983).

Fetal condylar chondrocytes have demonstrated precocious formation of matrix vesicles and accumulation of calcium intra and extracellularly at dexamethasone doses of 10^{-6} to 10^{-8} M (Lewinson and Silbermann, 1984). Supraphysiologic concentrations of dexamethasone in neonatal murine chondrocyte cultures results in a rapid calcium uptake within 2 hours and a decrease in tritiated thymidine uptake after a 24 hour lag period. The responses were protein and mRNA synthesis dependent (Maor and Silbermann, 1986). The genetic expression of elastic cartilage chondrocytes from rabbit ear cartilage was altered by

dexamethasone addition, which substantially increased the number of elastic fibers (Hinek et al., 1984).

D. Scientific Research Objectives.

The objectives of this study were to develope a system for normal chondrocyte calcification in vitro using the chondrocyte model of Boyan et al. (1988), to characterize the process both histologically and biochemically, and to examine the effect of dexamethasone on this system. The findings of others that dexamethasone may have significant time and dose related effects leaves many questions regarding the regulation of calcification. This study proposes to supply addition information.

II. METHODS

A. **Preparation of chondrocytes.** Costochondral cartilage from 125 g Sprague-Dawley rats was the source of resting and growth zone chondrocytes. The animals were sacrificed by CO_2 asphyxiation and their rib cages were removed by sharp dissection and placed in Dulbecco's modified Eagle's medium (DMEM) until the cartilages were removed. The resting and growth zone cartilages were carefully dissected out and slices incubated overnight at 37° C in 5% CO_2. The DMEM was then replaced by two 20 minute washes of Hank's balanced salt solution (HBSS), then 1 hour in 0.25% trypsin followed by 3 hours in 0.2% collagenase. After enzymatic digestion of the extracellular matrix, the debris was separated out by filtration using sterile 45 um mesh nylon and the cells were collected from the filtrate by centrifugation at 500 X g for 10 minutes. The cells were then resuspended in DMEM with 10% fetal bovine serum and plated in T-75 flasks at a density of 10,000 cells/cm^2 for resting zone cells and 25,000 cells/cm^2 for growth zone cells. Cells were grown to confluence and used in first and third passages during various fazes of experimentation.

B. **Effect of cell density.** When determining the best density in which to culture the cells for calcification, the cells were cultured 35 mm diameter tissue culture wells as follows for 40 days.

1. Resting zone chondrocytes at 10,000 cells/cm^2 and Growth zone chondrocytes at 25,000 cells/cm^2 in 2 ml of media (Boyan et al., 1988).

2. Resting zone chondrocytes and growth zone chondrocytes plated at 2 X 10^6 cells per 35 mm well which is 208,000/cm^2 in 2 ml of media (Vaananen et al., 1983).

3. Resting zone chondrocytes and growth zone chondrocytes, 5 X 10^5 cells in 0.1 ml of media plated in a 6 mm diameter removable cylinder within the 35 mm well which is 1,770,000/cm^2 (Fig 1). The cylinder was removed at 24 hrs and 2 ml of media added (Suzuki et al., 1981). 12 wells of each density of growth zone cells and 10 wells of each density of resting zone cells were plated.

C. **Effect of culture medium and medium supplements.** The cells were cultured using Dulbecco's modified Eagle's media (Gibco) with 10% fetal bovine serum and 50 ug/ml vitamin C, which was changed three times per week. To test the ability of various agents to increase calcification, either 3 mM or 10 mM beta-glycerolphosphate (BGP) was added to the media in some experiments. 1 mM ATP was added to some plates in one experiment to examine if ATP would increase Ca^{++} and Pi uptake in this system as had been reported in chick epiphyseal plate cultures (Chin et al., 1983). As mentioned in the introduction, dexamethasone has been shown by various authors (Bellows et al.,

1986; Bellows et al., 1987; Bellows et al., 1989; and Bellows and Aubin, 1989) to increase mineralization nodules in cultures of rat calvarial cells. Based on that information, 10^{-5} M to 10^{-9} M dexamethasone was added to the system in an attempt to increase the number of calcification nodules. The use of modified Biggers, Gwatkin, Judah (BGJ) media (Gibco) in other calcifying systems (Vaananen et al., 1983) led to an effort to examine its usefulness in this chondrocyte system.

D. **Protein content.** The protein content of each fraction was examined using the method of Helenius and Simons (1971) using bovine serum albumin as the standard.

E. **Enzyme activity.** Alkaline phosphatase activity in the cell layer was measured as a function of para-nitrophenol formation from para-nitrophenylphosphate (Bergmeyer, 1983), with cell free cultures run in parallel to assess the contribution of the fetal calf serum. This procedure was done in a 96 well plate as described by Hale et al. (1986) and the optical density (OD) read at 400nm on an automatic densitometer (Ebresol).

The alkaline phosphatase activity of both resting and growth zone chondrocytes was examined using various doses of dexamethasone on confluent cultures for 48 hours, and from the day of plating for 30 days.

F. **Light microscopy**. The presence of calcification nodules was determined by reverse-phase light photomicroscopy. Cultures were fixed in situ and stained using toluidine blue to visualize cartilage. Calcification was indicated by Von Kossa staining of cells cultured in the tissue slide chambers.

G. **Statistical analysis**. Student's t-test was used to examine the significance of differences in the biochemical data. All experiments were performed a minimum of three times. Each data point represents the mean + S.E.M. of at least 3 samples. Data are represented using a typical single experiment.

III. RESULTS

A. Chondrocyte culture cell density.

The first experiment examined whether plating density had any effect on the long term (30 days) potential of the culture to calcify in DMEM. Dissection of 24 rats, yields approximately 23 X 10^6 growth zone cells at confluence in primary culture. Since only 11 experimental cultures could be used when plated at a density of 2 X 10^6 cells per 35 mm diameter well (Vaananen et al., 1983), we determined if the lower density of 9.6 X 10^4 growth zone cells per well (Boyan et al., 1988) would produce calcification nodules in culture. Cells were plated into 35 mm diameter plastic tissue culture wells in the three densities described previously.

When examined under reverse-phase microscopy and with toluidine blue staining, the different density cultures all became confluent at 5-7 days but by different means. Wells plated with 2 X 10^6 cells per well (Vaananen et al., 1983) had large aggregations of floating cells with areas of detaching cells during the first 3-5 days but all areas of the well were covered with cells at 7 days. Wells plated using 5 X 10^5 cells in the 6 mm cylinder (Plate 1) (Suzuki et al., 1981) had detaching areas and floating cells from the center for 3-5 days after the cylinder was removed and then took 7 days to grow to the edge of the plate from the densely plated central area. At

30 days the cells were similar regardless of initial plating density. The chondrocyte cultures appeared to go through four phases of maturation described below.

1. Confluency. At day 7 wells of both cell types became confluent. The resting zone cells were densely packed and fibroblast-like and growth zone cells were polygonally shaped in most of the well (Plate 2) with spindle shapes at the curved periphery of the well.

2. Large stellate cells. At days 5-10, some cells became large with a stellate shape. The cells were as much as ten times larger than the cells of the densely packed mass and often formed clusters. The stellate arms of one large cell often contacted another. Although these cells were numerous, not all cells became enlarged. Resting zone cells tended to have fewer large cells even at 10 days than was seen in 7 day growth region cultures. The large cells constituted only a small percentage of the total cells but occupied substantial area in the well. The nucleus of these large cells became quite distinct with a granular cytoplasm. The cells appeared to be producing a matrix (Plates 3 and 4).

3. Nodule formation. Nodules similar to those described by Bellows and co-workers (1986) were first observed from 10-14 days in growth zone cultures. The nodules were dense clusters of cells with larger stellate cells surrounding and often had one or more large cells centrally located. The size of the

Plate 1.

35 mm tissue culture well with 6 mm diameter cylinder sealed in place.

Plate 2.

Confluent third passage resting zone cells at 7 days in culture. Reverse phase microscopy, magnification 10X.

Plate 3.

Large stellate third passage growth zone cells at 10 days which appear to be producing a product. Reverse phase microscopy, magnification 10X.

Plate 4.

Large stellate primary cell culture 12 day growth zone cells with granular cytoplasm and distinct nucleus. Reverse phase microscopy, magnification 10X.

nodules varied widely and were frequently connected which made exact counting and determination of surface area difficult. Initially the nodules were small (Plate 5) but increased in numbers and size (Plates 6 and 7).

4. Calcification. Using the Von Kossa stain for calcification, areas around and within the nodules demonstrated the dark granules consistent with calcification (Plate 8). Calcification was first seen as early as 14 days but was more common at 21 days. Not all nodules showed calcification and the amount of calcification within the nodules that did show mineralization was varied.

B. **Media.**

Resting and growth zone cells both failed to grow to confluence using BGJ media with 10% fetal bovine serum. Growth zone cells grew well in DMEM producing densely packed confluent cultures (Plate 9). When grown in BGJ, growth zone chondrocytes failed to thrive and most cells were dead and detached at two weeks (Plate 10). Resting zone chondrocytes, as was noted in the density experiment and repeated in the media experiment, grew to confluency in DMEM (Plate 11) but populated the wells only sparsely when BGJ was used (Plate 12). At 20 days there were so few attached cells that the use of BGJ was not considered useful in the system and the cultures were discarded.

Plate 5.

Initial nodule formation in primary culture 10 day growth zone cells. Reverse phase microscopy, magnification 4X.

Plate 6.

Nodule formation with dense clusters of cells oriented toward the nodule. 12 day primary culture growth zone cells. Reverse phase microscopy, magnification 10X.

Plate 7.

Large mature nodule with a centrally located cluster of round cells. 16 day primary culture growth zone cells. Reverse phase microscopy, magnification 10X.

Plate 8.

Calcification seen in a nodule of cultured 21 day primary culture growth zone cells. Von Kossa staining, magnification 10X.

Plate 9.

Confluent 7 day third passage growth zone cells cultured in DMEM media with 10% fetal bovine serum. Reverse phase microscopy, magnification 10X.

Plate 10.

Third passage growth zone cells cultured in BGJ media with 10% fetal bovine serum failed to thrive and were almost completely detached by 14 days in culture. Reverse phase microscopy, magnification 10X.

Plate 11.

Third passage 7 day resting zone cells cultured to confluence in DMEM media with 10% fetal bovine serum. Reverse phase microscopy, magnification 10X.

Plate 12.

Third passage resting zone chondrocytes cultured in BGJ media with 10% fetal bovine serum failed to thrive with very few attached cells at 14 days. Reverse-phase microscopy, magnification 20X.

C. **Effect of ATP.**

When added to DMEM at 1 mM a concentration, ATP had a significantly negative effect on both resting and growth zone cultures. Large aggregations of floating cells detached from the wells with no repopulation of the areas and only a few cells survived at five days (Plate 13). By 10 days the wells were barren and discarded.

D. **Effect of beta-glycerol phosphate.**

When examined in the chondrocyte culture system at 3 mM and 10 mM concentrations added to DMEM with 10% fetal bovine serum, BGP had no observed effect on the morphology of cultures and their ability to calcify when compared to cells grown without BGP.

E. **Effect of dexamethasone.**

Dexamethasone (10^{-7}M) appeared to decrease the time needed for each stage of chondrocyte maturation. Nodule formation was dramatically increased in an accelerated time frame for both resting and growth zone cells with the resting cells maturing a few days slower. Growth zone cells grown with dexamethasone formed nodules by 5-7 days. Growth zone cells cultured without dexamethasone showed some large stellate cells and occasional nodules by 14 days (Plate 14). However, when growth zone chondrocytes were cultured in parallel with dexamethasone added,

Plate 13.

Primary culture growth zone chondrocytes cultured in DMEM media with 10% fetal bovine serum and 1 mM ATP at 7 days. ATP caused large sheets of cells to detach from the culture wells. Reverse-phase microscopy, magnification 10X.

Plate 14.

Primary culture growth zone chondrocytes cultured without dexamethasone at 14 days demonstrate some large cells and small nodule clusters. Toluidine blue staining, magnification 10X.

the number of large stellate cells and nodules was dramatically increased at 14 days (Plate 15). Von Kossa staining of 14 day cultures of growth zone cells without dexamethasone demonstrated little sign of calcification (Plate 16), but the cells cultured with dexamethasone had numerous stained areas consistent with calcification (Plate 17).

Resting zone chondrocytes cultured without dexamethasone demonstrated few large cells in 14 day confluent cultures (Plate 18) and infrequent nodules at 21 days. However, the addition dexamethasone altered the cellular morphology in such a way that 14 day resting zone cells cultured with the steroid resembled 14 day growth zone cells grown without the steroid, and contained increased numbers of large cells and occasional nodules (Plate 19). Dexamethasone appeared to accelerate maturation and increase cell differentiation to cells that would form nodules. Nodule associated punctate calcifications were observed to peak at 20-24 days as demonstrated by Von Kossa staining of growth zone cells at 20 days (Plate 20). Observations in the early morphology experiments that the large cells were producing a matrix, were substantiated by Trichrome collagen staining of 20 day growth zone cells cultured with dexamethasone. Areas of collagen around the large stellate cells were observed using this stain (Plate 21).

Plate 15.

Primary culture growth zone chondrocytes cultured with 10^{-7}M dexamethasone at 14 days demonstrating increased numbers of large cells and nodules. Toluidine blue staining, magnification 10X.

Plate 16.

Von Kossa calcification staining of 14 day primary growth zone cultures without dexamethasone demonstrate little sign of calcification. Magnification 10X.

Plate 17.

Von Kossa calcification staining of 14 day primary growth zone cultures with dexamethasone exhibiting numerous areas of calcification. Magnification 10X.

Plate 18.

Resting zone cells at 14 days in primary culture without dexamethasone, demonstrate few large cells or nodules. Toluidine blue staining, magnification 10X.

Plate 19.

Resting zone primary culture with 10^{-7}M dexamethasone added at 14 days demonstrating increased numbers of large cells and nodules. Toluidine blue staining, magnification 10X.

Plate 20.

Growth zone primary culture with 10^{-7}M dexamethasone added at 20 days demonstrating perinodular calcification. Von Kossa staining, magnification 20X.

Plate 21.

Primary growth zone cell culture with 10^{-7}M dexamethasone added, showing perinodular matrix production at 20 days. Trichrome stain, magnification 10X.

F. **Alkaline phosphatase activity.**

Incubation with dexamethasone altered alkaline phosphatase activity in both short (0-2 days) and long (6-30 days confluent) term cultures.

1. Short term effects of dexamethasone. The short term time course data on growth zone cells using 10^{-6} and 10^{-7}M dexamethasone demonstrates a time and dose response (Fig. 1). At 6 hours the cultures treated with 10^{-6}M dexamethasone showed significantly increased alkaline phosphatase activity compared to the untreated controls, and at 24 and 48 hrs both the 10^{-6} and 10^{-7}M concentrations demonstrated significantly higher activity. Higher levels of activity were consistently observed in growth zone cultures compared to resting zone cultures. This was statistically significant at 10^{-6}M dexamethasone.

The results of short term time course experiments on resting zone cultures yielded results similar to those seen in growth zone cells. Dexamethasone concentrations of 10^{-6} and 10^{-7}M significantly increased activity compared to controls, and the increase was seen as early as 60 minutes (Fig. 3) in the resting cells compared to 6 hours in the growth zone cells. Dose response data gathered at 48 hrs (Fig. 2) demonstrated a significantly increased level of alkaline phosphatase activity which was 2-4 times greater than controls using concentrations of 10^{-8} to 10^{-6}M dexamethasone in both resting and growth zone cultures.

Figure 1.

Short term effect of dexamethasone on alkaline phosphatase activity in growth zone chondrocyte cultures.

Each symbol indicates mean values $\pm$ S.E.M. of 6 or more samples and * indicates significance at the $p<0.05$ level.

Confluent, third passage cultures were incubated with 10^{-7}M dexamethasone for 48 hours. Data are presented from a single experiment and represent results typical of 2 additional replicate experiments.

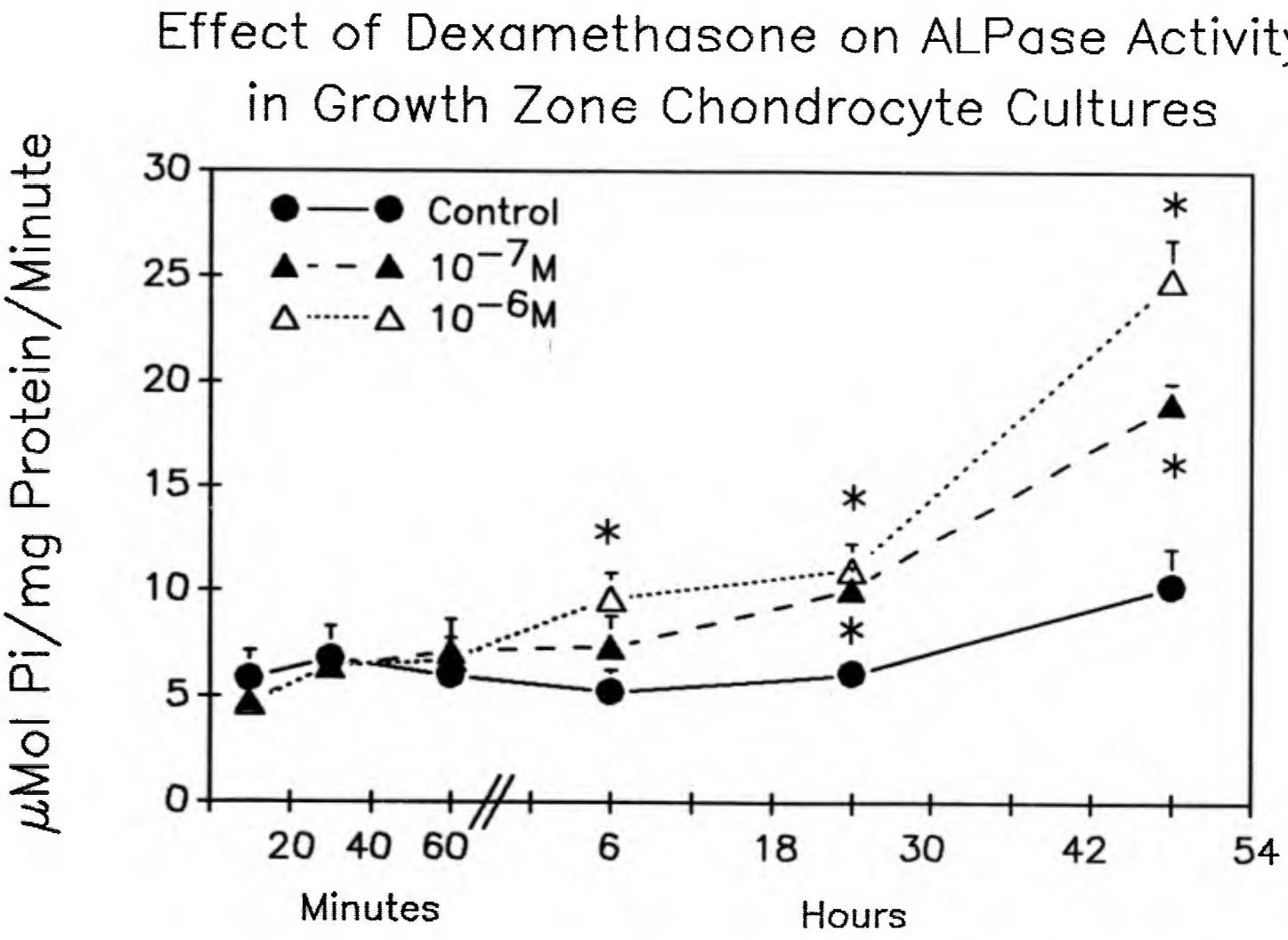

Effect of Dexamethasone on ALPase Activity
in Growth Zone Chondrocyte Cultures
μMol Pi/mg Protein/Minute
30
25
20
15
10
5
0
Control
10⁻⁷M
10⁻⁶M
20
40
60
6
18
30
42
54
Minutes
Hours

Figure 2.

Effect of dexamethasone on alkaline phosphatase activity after 48 hours on resting zone (RC) and growth zone (GC) chondrocytes.

Each symbol shows the mean values $\pm$ S.E.M. of 6 or more samples. * indicates significance at the $p<0.05$ level.

Confluent, third passage cells were incubated with 10^{-7}M dexamethasone for the time points shown. Data presented are from one of three replicate experiments.

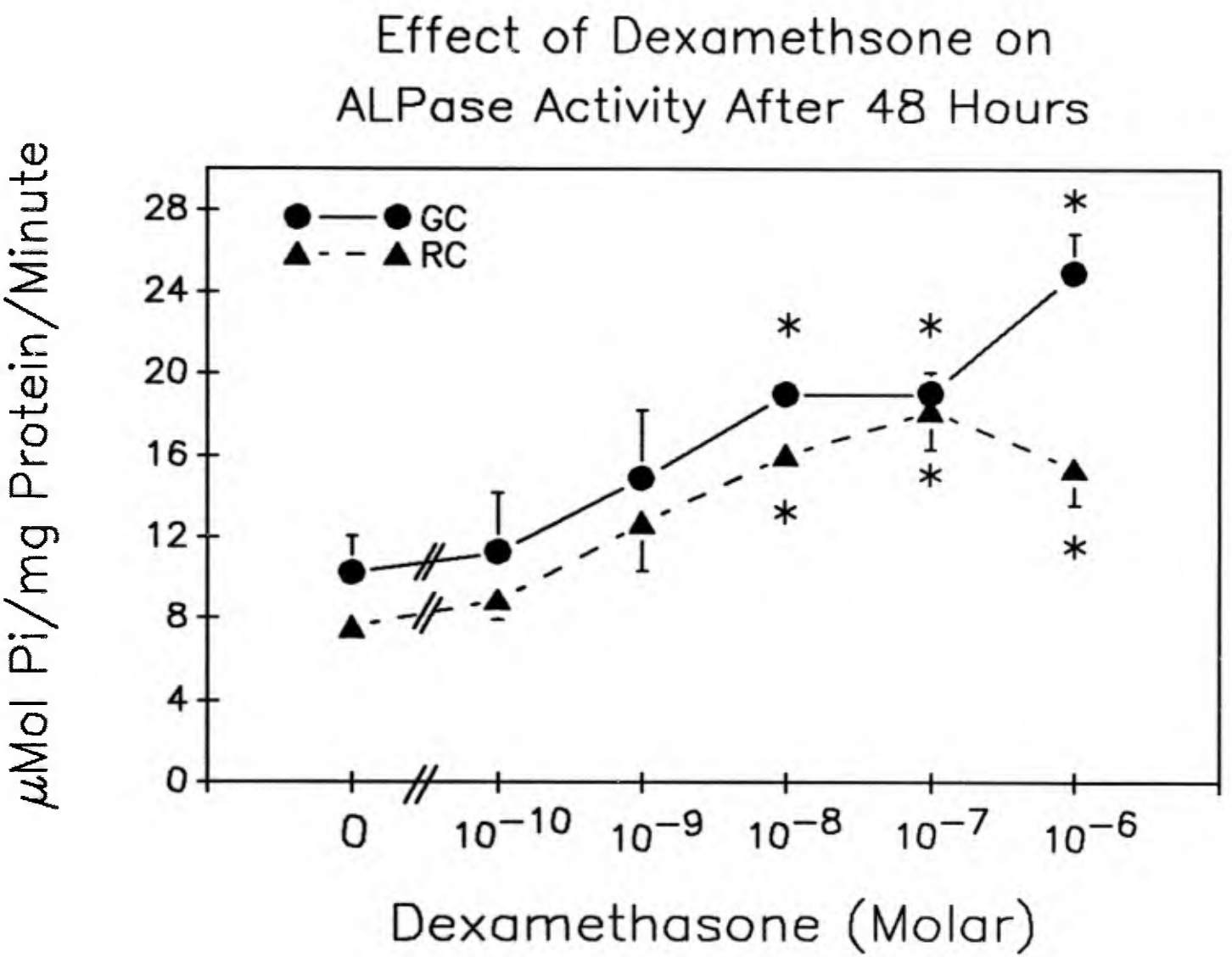
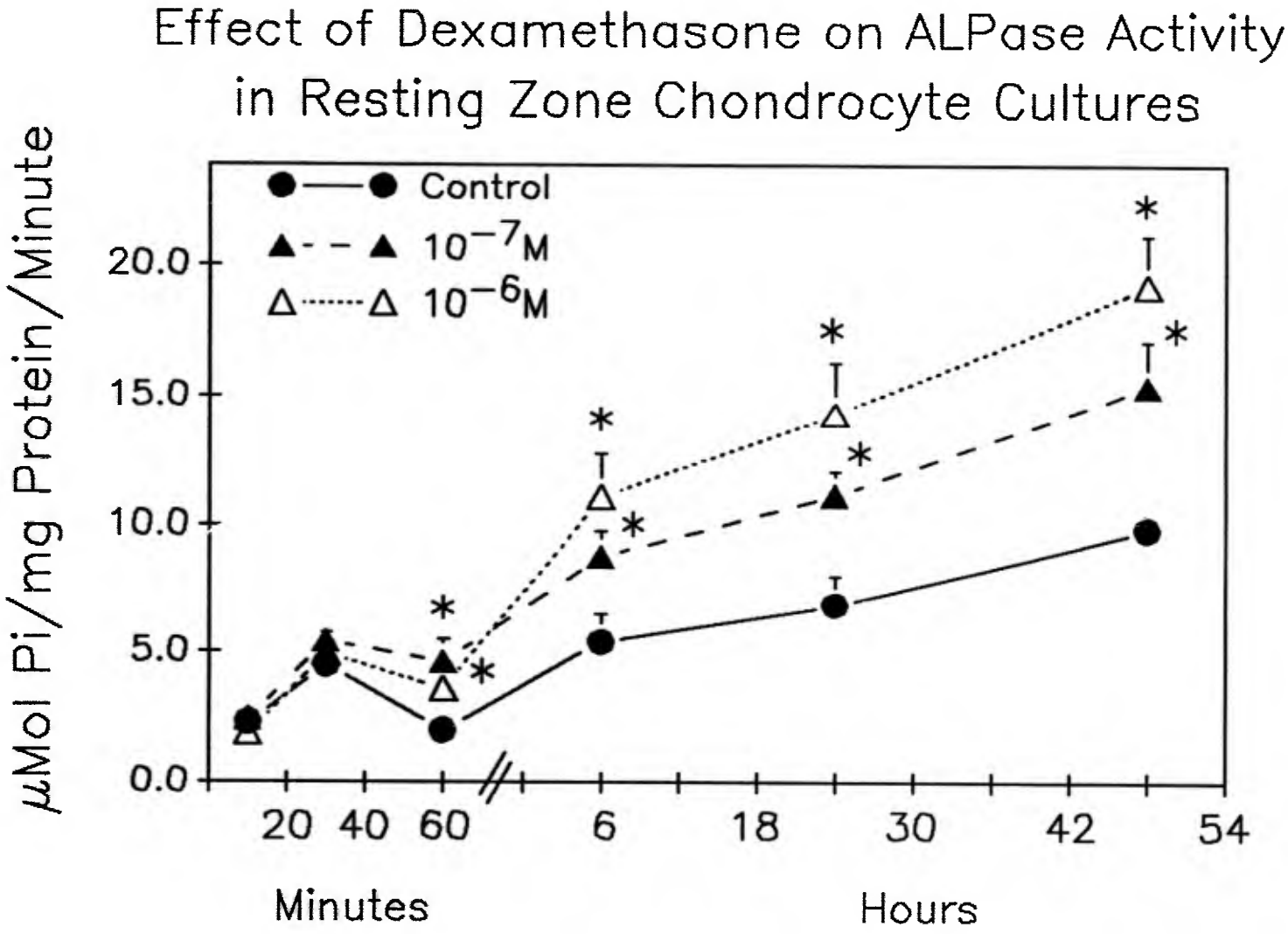

Effect of Dexamethsone on
ALPase Activity After 48 Hours
μMol Pi/mg Protein/Minute
28
24
20
16
12
8
4
0
GC
RC
*
0
10−10
10−9
10−8
10−7
10−6
Dexamethasone (Molar)

Figure 3.

Effect of dexamethasone on alkaline phosphatase activity in resting zone chondrocyte cultures from 0 to 48 hours.

Each symbol shows the mean values ± S.E.M. of 6 or more samples. * indicates significance at the $p<0.05$ level.

Confluent, third passage cells were incubated with 10^{-6}M and 10^{-7}M dexamethasone for the time points shown. Data presented are from one of three replicate experiments.


Effect of Dexamethasone on ALPase Activity
in Resting Zone Chondrocyte Cultures
μMol Pi/mg Protein/Minute
Control
10⁻⁷M
10⁻⁶M
20.0
15.0
10.0
5.0
0.0
20
40
60
6
18
30
42
54
Minutes
Hours

2. Long term effect of dexamethasone.

When the growth zone cultures were examined from 6-30 days, a 4-5 fold increase in activity was observed at days 12, 18, and 24, with a sharp decrease at day 30 (Fig. 4). The addition of 10^{-7}M dexamethasone inhibited the increase in activity throughout the time course, although activity did continue to rise between days 6 and 30. At day 30 the activity was similar for the dexamethasone treated cultures and the control cultures.

Resting zone cultures examined for 6-30 days behaved similarly to the growth zone cultures. Resting zone controls demonstrated a 10 fold increase in activity from day 6 to day 24 with a decrease at day 30 (Fig. 5). The dexamethasone treated cells remained significantly lower than controls at days 12, 18, 20, 24, and 30.

When the alkaline phosphatase activity was examined in dexamethasone treated cultures from the day of plating, there was a lower level of activity in both cell types. In growth zone cultures treated with dexamethasone, activity was significantly lower compared to controls (Fig. 6). Similar results were seen when resting zone cells were examined (Fig. 7) but the reduced level of activity was more pronounced with 10^{-6}M dexamethasone.

A dose response reduction of alkaline phosphatase activity by dexamethasone was observed in growth (Fig. 8) and resting zone cultures (Fig. 9) from 12 to 30 days. In growth zone

Figure 4.

Effect of dexamethasone on alkaline phosphatase activity in growth zone chondrocyte cultures from 6 to 30 days.

Each symbol shows the mean values ± S.E.M. of 6 or more samples. * indicates significance at the $p<0.05$ level.

Confluent, third passage cells were incubated with 10^{-7}M dexamethasone for the time points shown. Data presented are from one of three replicate experiments.

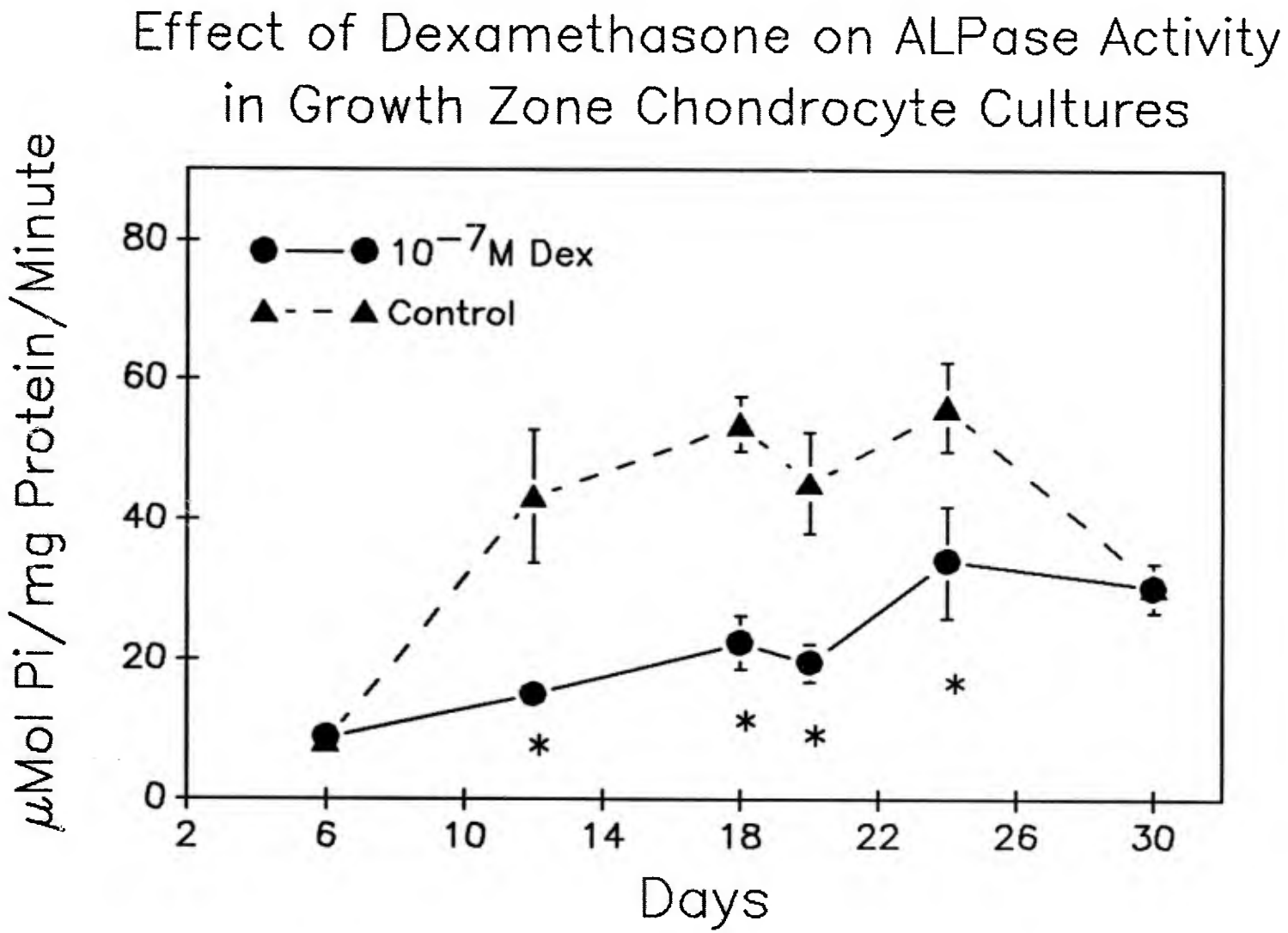

Effect of Dexamethasone on ALPase Activity
in Growth Zone Chondrocyte Cultures
μMol Pi/mg Protein/Minute
80
60
40
20
0
10⁻⁷M Dex
Control
2
6
10
14
18
22
26
30
Days

Figure 5.

Effect of dexamethasone on alkaline phosphatase activity in resting zone chondrocyte cultures from 6 to 30 days.

Each symbol shows the mean values ± S.E.M. of 6 or more samples. * indicates significance at the $p<0.05$ level.

Confluent, third passage cells were incubated with 10^{-7}M dexamethasone for the time points shown. Data presented are from one of three replicate experiments.

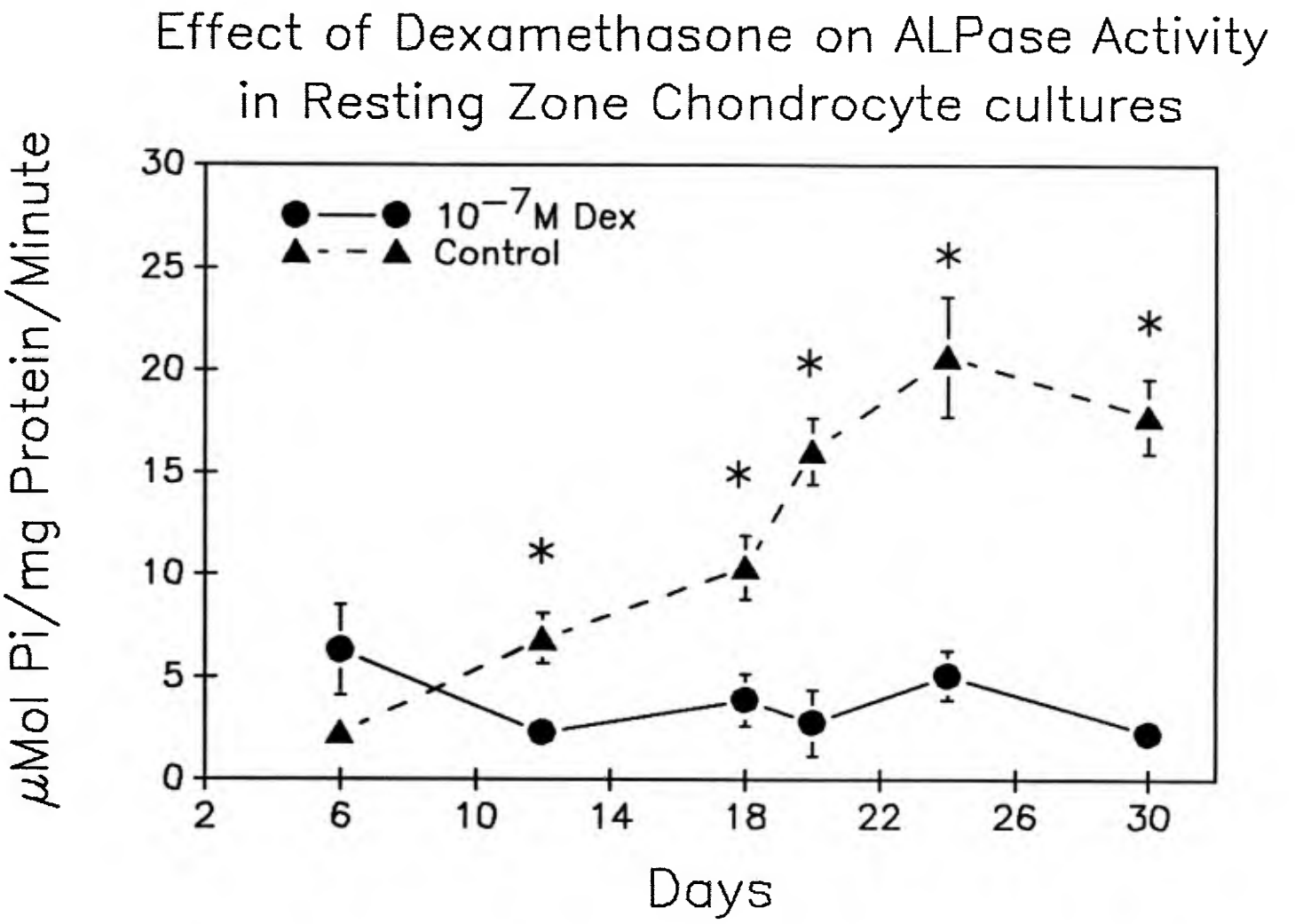

Effect of Dexamethasone on ALPase Activity
in Resting Zone Chondrocyte cultures
μMol Pi/mg Protein/Minute
30
25
20
15
10
5
0
2
6
10
14
18
22
26
30
Days
10⁻⁷M Dex
Control
*
*
*
*
*

Figure 6.

Long term effect of dexamethasone on alkaline phosphatase activity in growth zone chondrocyte cultures from 1 to 30 days.

Each bar represents the mean value ± S.E.M. of 6 or more samples and * indicates significance at the $p<0.05$ level.

Primary cultures were incubated in medium containing 10^{-6}M and 10^{-7}M dexamethasone for 30 days. Cultures were harvested at the time points shown. Data presented are from one of three replicate experiments.

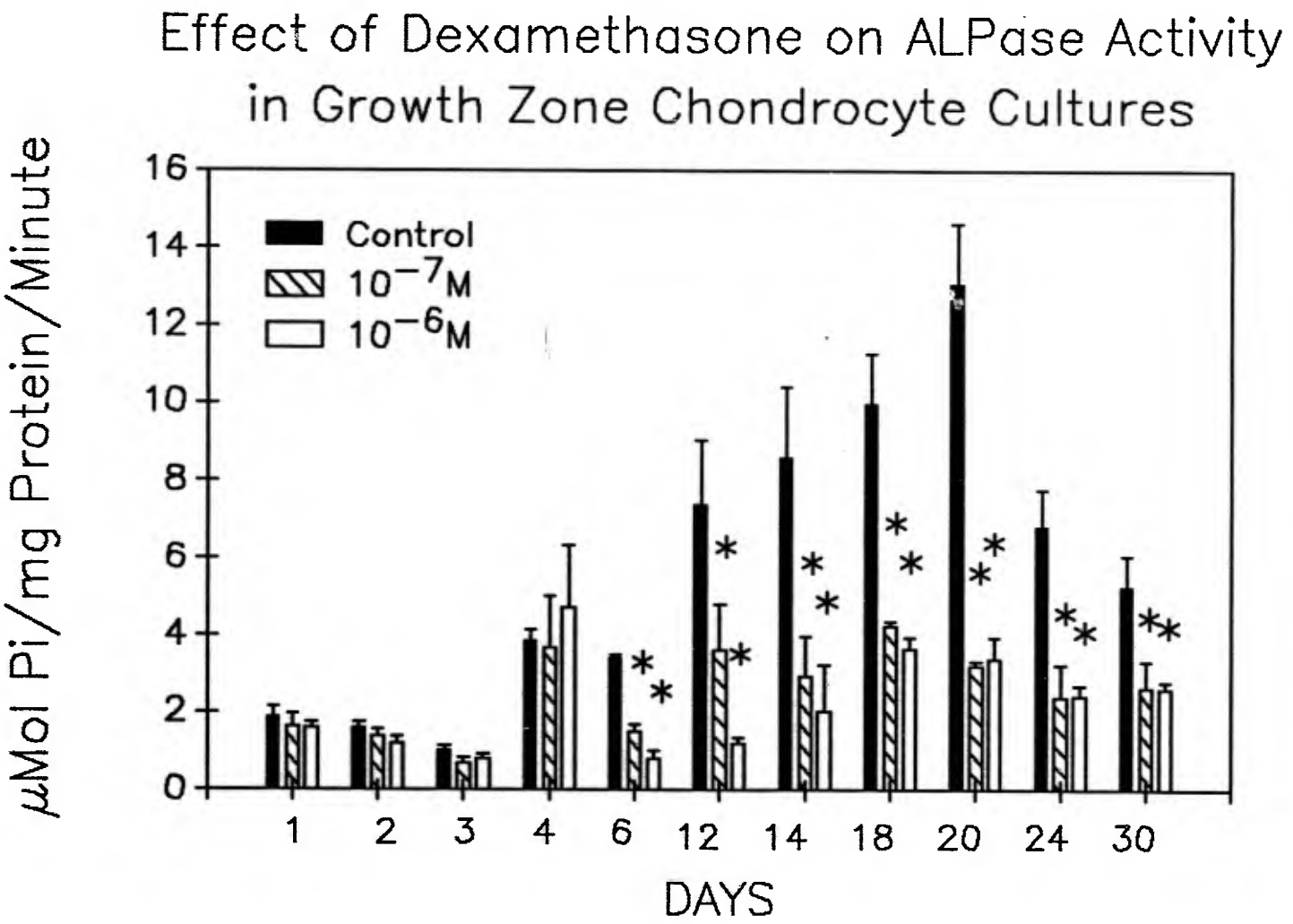

Effect of Dexamethasone on ALPase Activity
in Growth Zone Chondrocyte Cultures
μMol Pi/mg Protein/Minute
16
14
12
10
8
6
4
2
0
Control
10⁻⁷M
10⁻⁶M
1
2
3
4
6
12
14
18
20
24
30
DAYS

Figure 7.

Long term effect of dexamethasone on alkaline phosphatase activity in resting zone chondrocyte cultures from 1 to 30 days.

Each bar represents the mean value $\pm$ S.E.M. of 6 or more samples and * indicates significance at the $p<0.05$ level.

Primary cultures were incubated in medium containing 10^{-6}M and 10^{-7}M dexamethasone for 30 days. Cultures were harvested at the time points shown. Data presented are from one of three replicate experiments.

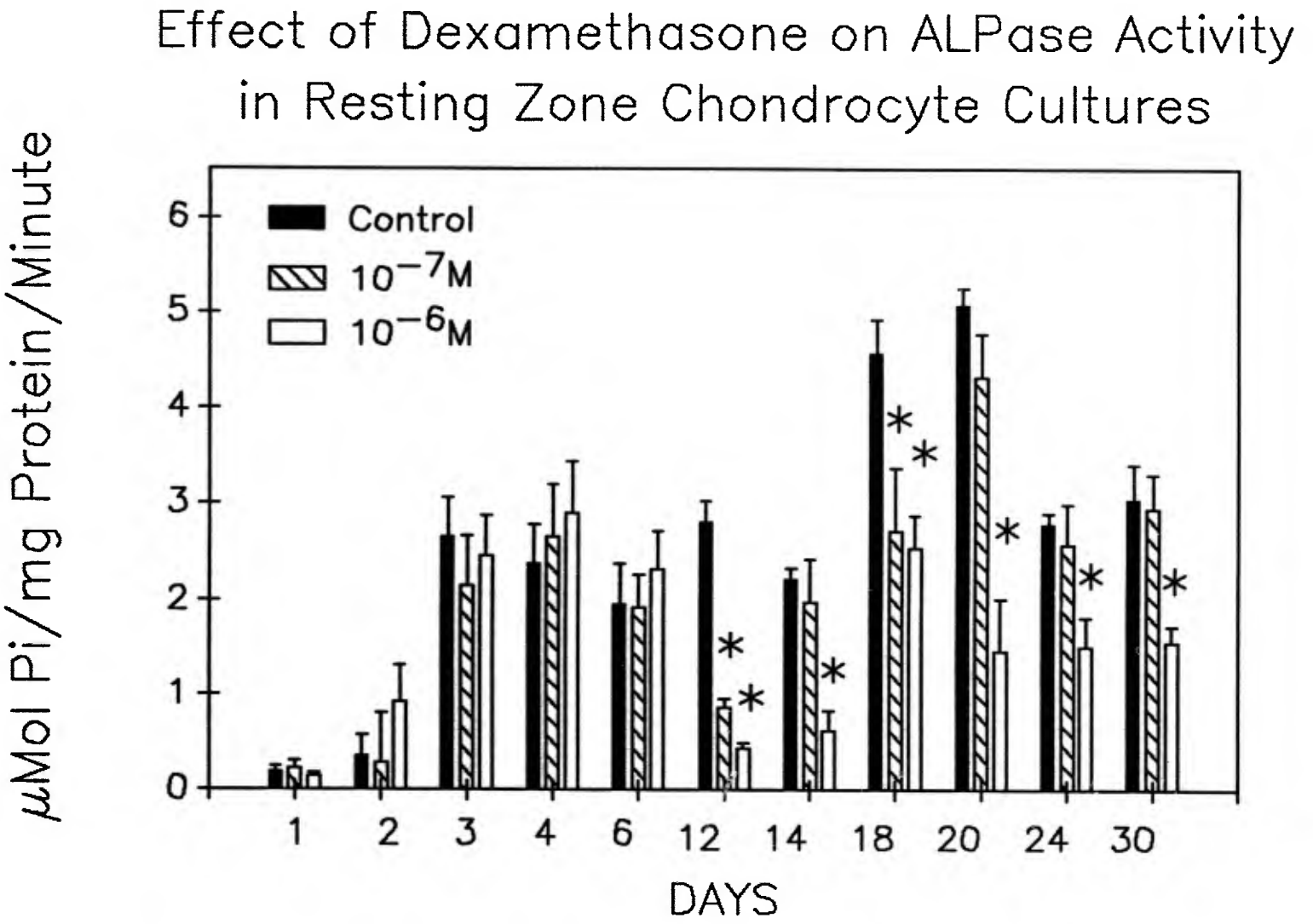

Effect of Dexamethasone on ALPase Activity
in Resting Zone Chondrocyte Cultures
μMol Pi/mg Protein/Minute
Control
10⁻⁷M
10⁻⁶M
0
1
2
3
4
5
6
1
2
3
4
6
12
14
18
20
24
30
DAYS

Figure 8.

Long term effect of dexamethasone on alkaline phosphatase activity in growth zone chondrocyte cultures: Dose response from 4 to 30 days.

Each bar represents the mean value ± S.E.M. of 6 or more samples and * indicates significance at the $p<0.05$ level.

Primary cultures were incubated with 10^{-6}M to 10^{-9}M dexamethasone for 30 days. Cultures were harvested on the time points indicated. Data presented are from one of three replicate experiments.

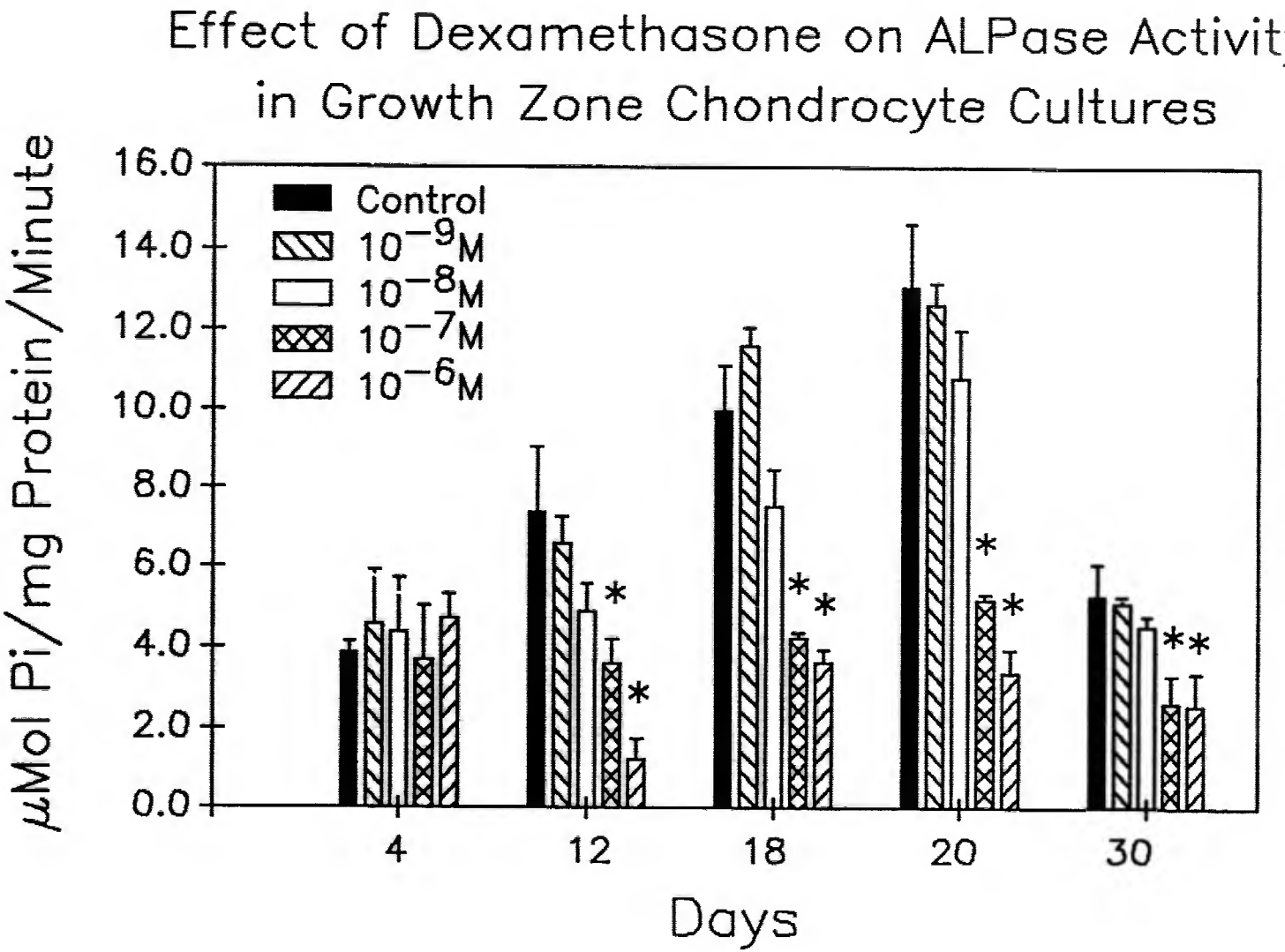
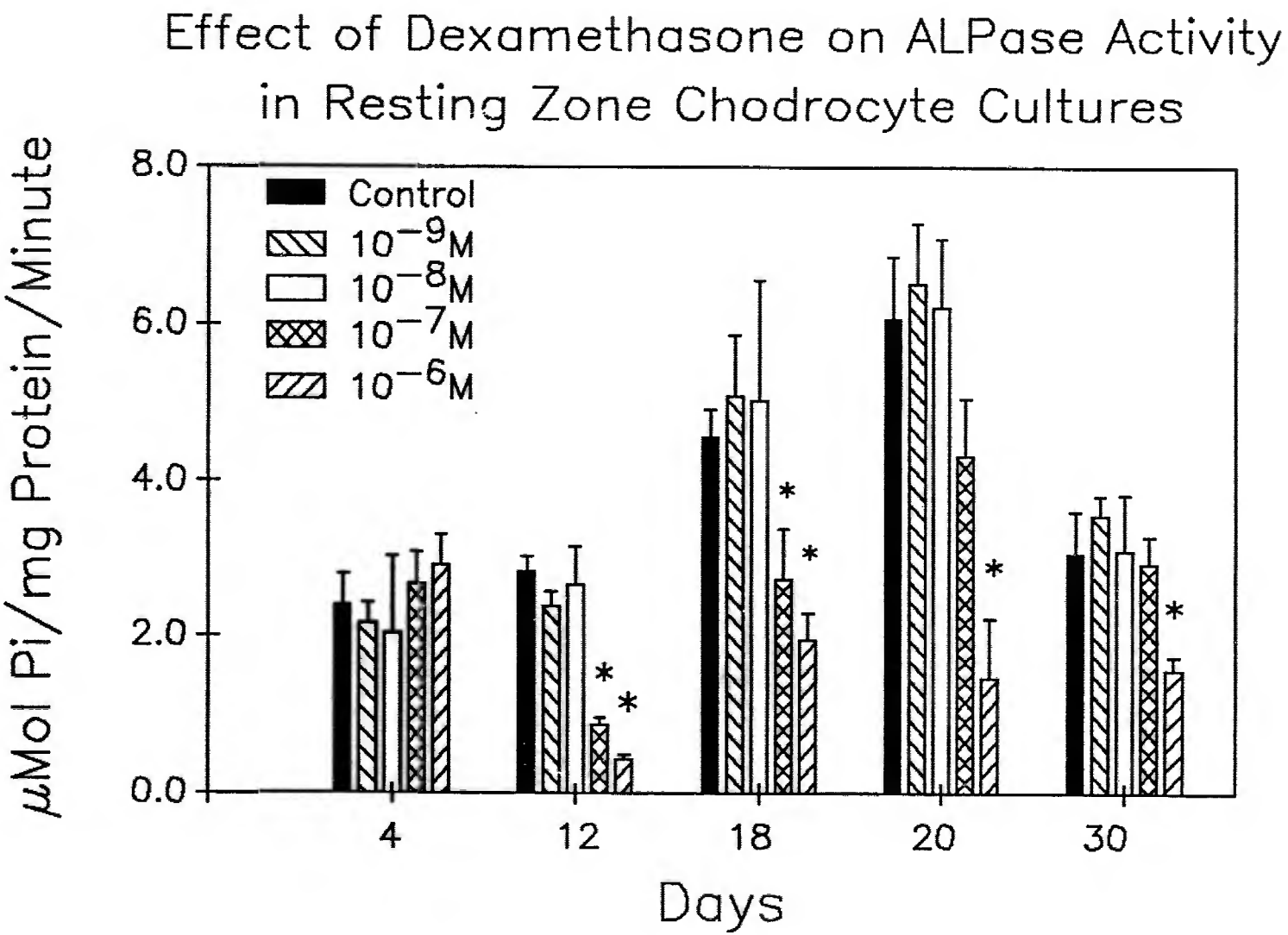

Effect of Dexamethasone on ALPase Activity
in Growth Zone Chondrocyte Cultures
µMol Pi/mg Protein/Minute
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0.0
Control
10⁻⁹M
10⁻⁸M
10⁻⁷M
10⁻⁶M
4
12
18
20
30
Days

Figure 9.

Long term effect of dexamethasone on alkaline phosphatase activity in resting zone chondrocyte cultures: Dose response from 4 to 30 days.

Each bar represents the mean value ± S.E.M. of 6 or more samples and * indicates significance at the $p<0.05$ level.

Primary cultures were incubated with 10^{-6}M to 10^{-9}M dexamethasone for 30 days. Cultures were harvested on the time points indicated. Data presented are from one of three replicate experiments.


Effect of Dexamethasone on ALPase Activity
in Resting Zone Chodrocyte Cultures
μMol Pi/mg Protein/Minute
8.0
6.0
4.0
2.0
0.0
Control
10⁻⁹M
10⁻⁸M
10⁻⁷M
10⁻⁶M
*
4
12
18
20
30
Days

cells, 10^{-7} and 10^{-6}M concentrations both resulted in significantly reduced levels of activity compared to controls from 12 to 30 days (Fig. 8) with lower concentrations failing to demonstrate any effect. A similarly reduced activity level was seen in resting zone cultures with 10^{-7}M dexamethasone reducing activity at days 12 and 18, and 10^{-6}M concentration lowering activity at days 12, 18, 24, and 30.

IV. DISCUSSION

A. In vitro chondrocyte model.

The model developed for this project evolved from a series of experiments which examined variables which previous studies on in vitro chondrocyte calcification had introduced (Suzuki et al., 1981; Vaananen et al., 1983). Although calcification had been reported by these authors, there were no reports of calcification in cultures of normal chondrocytes in the absence of media supplements or other cell types. The results of this study demonstrate clearly that normal chondrocytes will undergo chondrogenic differentiation in culture and calcify independent of an additional phosphate or agents which promote differentiation.

In the study by Vaananen et al. (1983), the chondrocytes had been isolated from the growth plates of rachitic rats. This strategy increased the yield of uncalcified cartilage but the cells in the zone which would have been calcified may not reflect the behavior of normally maturing chondrocytes. The methodology of this group included plating the cells at a density of 200,000 chondrocytes/cm^2 in order to form a multilayer. In a series of experiments using normal chondrocytes, this high plating density had no beneficial effect on the maturation of the cultures to confluence and beyond, and consumed large numbers of cells needlessly when compared to a density which was ten times less.

Co-culturing chondrocytes with bone marrow cells as described by Suzuki et al. (1981), added a variable which left questions regarding the potential of chondrocytes to calcify without bone marrow co-culturing. Their culturing technique involved plating the chondrocytes in a small cylinder within a tissue culture well at a density of 1.7 million cells/cm^2 for 24 hours and then co-culturing the chondrocytes with the marrow cells. The high plating density was intended to form a multilayer, but the experiments done as part of the present study showed that most of the cells sloughed off the plate in the first few days, and maturation of the wells to confluence was not enhanced.

Mineralization was observed by Von Kossa stain as early as 10 days in growth zone cultures, but was more common and abundant at 21-30 days. Previous work by other authors using osteoblast cultures (Ecarot-Charrier et al., 1983), has implied that the media needed supplementation of some sort in order for the cultures to calcify. In their study, the cells were only examined up to 14 days, and the time frame may have been too short to allow for natural differentiation of the cells to form nodules and calcify. The assertion of these authors that 5-10 mM BGP needed to be added for calcification was tested in the present system and shown not to be the case.

In any case, the type of mineral deposit that is seen when BGP is added has recently been shown to differ from normal

mineralization in vivo and the increase in calcified area seen in organ culture with BGP added is mainly ectopic (Gronowicz et al., 1989). The same process of ectopic calcification may explain the increase calcium and phosphate uptake seen in chick epiphyseal plate organ culture when 1 mM ATP is added (Chin et al., 1983). The increase in phosphate contributed by the ATP in the present system may have accounted for the toxic effect of the ATP on the cells and is similar to the effect observed by Gronowicz and co-workers who observed dystrophic calcification and ultrastructural alterations of some mitochondria.

Morphologically, the calcification nodules seen in our experiments are indistinguishable from those previously described by Bellows and co-workers (1986) which have been shown to have hydroxyapatite within (Bhargava et al., 1988). The mineralization associated with these nodules is punctate when observed by light microscopy (plate 20), and differs from the large ectopic crystals present when additional phosphate is added.

The formation of the nodules and their mineralization follows a sequence of maturation in the system which is very similar to chondrocyte maturation seen in vivo (Brighton and Hunt, 1978). In vivo, the cells from the growth zone adjacent to the resting zone go through proliferative and hypertrophic phases before maturation and calcification. After plating there is proliferation of the chondrocytes until the well becomes

confluent at which time some clusters of the cells become hypertrophic and nodules begin to form (plate 5).

B. **Effect of dexamethasone on the model.**

Dexamethasone increased the number of nodules seen and this is consistent with the findings of other authors in osteoblast systems (Bellows et al., 1986, Bellows et al., 1987). Although the nodules were not quantified in the present study there was an obvious increase (plates 14 and 15) when dexamethasone was added. In the rat calvarial osteoblast model, each nodule appears to have one progenitor cell and the number of progenitor cells can be increased from 1/340 to 1/225 with the addition of dexamethasone (Bellows and Aubin, 1989). The observation of nodules earlier when dexamethasone is added, also agrees with the findings of Bellows and co-workers (1986). The effect of dexamethasone on cell proliferation is of interest in the present study and could have effected the rate of maturation. The findings of Chen and co-workers (1983) indicating that densely packed rat calvarial cells were stimulated to proliferate by dexamethasone, while in sparse cultures proliferation was inhibited, may explain the increase in hypertrophic cell maturation observed in post-confluent cultures treated with dexamethasone.

Alkaline phosphatase activity is increased in the growth plate as zone of calcification is approached and is increased in

matrix vesicles. Bellows et al. (1986) demonstrated that alkaline phosphatase staining of osteoblast cultures is very concentrated in the nodules formed by osteoblast cultures, indicating that it is associated with differentiation and calcification here as well. In the untreated cultures examined in our study, alkaline phosphatase activity increased as nodules developed and peaked as mineral deposition occurred.

Our finding that dexamethasone initially stimulated and then inhibited alkaline phosphatase activity is similar to the finding of Canalis (1983). In his rat calvarial cultures, dexamethasone stimulated type I collagen formation and alkaline phosphatase activity initially, but then inhibited both by decreasing cell proliferation in the long term. This suggests that dexamethasone may have two effects. One is to inhibit proliferation, and the other is to stimulate differentiation.

It has been shown, that as mineralization begins there is an abrupt decrease in type I collagen synthesis and matrix formation in the chick periosteal model (Tenenbaum et al., 1990). The optimal level for alkaline phosphatase activity associated with calcification is not known, but a similar post-calcification decrease may also be active. The control growth zone cultures calcify most often at 21-30 days, and it is soon after that the levels of alkaline phosphatase activity decrease (Fig. 6).

It is known that the receptors for steroids decrease in number as cells mature through the growth zone palisade to the zone of initial calcification (Kan et al., 1984), but the activity of these receptors may be increased so the overall effect on the cells is not clear. A possible explanation for the increase in calcification at an early time frame, may be consistent with the findings of Lewinson and Silbermann (1984). They showed that concentrations of dexamethasone identical to those used in the present study stimulated a marked precocious formation of matrix vesicles.

The data from this study suggest a potential beneficial effect on endochondral bone formation associated with fracture healing, by increasing the differentiation of cells capable of forming calcification nodules. Further studies, to determine the appropriate dose of dexamethasone and time period, in a non-union animal model, may determine if there is a clinical application for the findings of this study.

V. SUMMARY

Dexamethasone alters the morphology of chondrocyte cultures, increasing the number of calcification nodules in a manner similar to that seen in osteoblast cultures. The calcification is nodule-associated as demonstrated by Von Kossa stain, and is composed of small punctate granules within the nodule, and not large randomly distributed crystals as can be found in cultures heavily supplemented with phosphate.

Dexamethasone has a significant stimulatory effect on the alkaline phosphatase activity for 48 hours in both resting and growth zone confluent cultures, and a significant inhibitory effect beyond 6 days from the day of plating. These effects are dose dependent and the doses of 10^{-6} and 10^{-7}M had the largest inhibitory effects.

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