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Dexamethasone Stimulates Osteogenic Differentiation in Vertebral and Femoral Bone Marrow Cell Cultures: Comparison of IGF-I Gene Expression

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Abstract Osteoblast-like cell cultures have been established from the marrow of adult rat vertebrae. We have simultaneously examined the response to dexamethasone (dex) treatment in cultures of young adult female vertebral and femoral marrow cells. Alkaline phosphatase (AP) activity was analyzed as well as the expression of mRNAs for osteocalcin (OC) and insulin-like growth factor I (IGF-I). The vertebral and femoral marrow cells were maintained for 7 days in primary culture with or without 10^{-8} M dex and then 6 days in secondary culture without dex or with 10^{-8} M or 10^{-7} M dex. All cells were examined on day 6 of secondary culture. Vertebral and femoral cultures each expressed the highest AP enzyme levels when grown with dex in primary culture (10^{-8} M) and secondary culture (10^{-7} M). Under all experimental conditions, vertebral cultures had lower AP enzyme activity than femoral cultures. When dex was omitted from secondary culture, OC gene expression was not detected in either vertebral or femoral passaged cells even if dex was present in primary culture. For dex conditions where OC was expressed, vertebral cultures had higher OC mRNA steady-state levels than femoral cultures. IGF-I gene expression was detected by Northern analysis in both vertebral and femoral secondary cultures. However, vertebral marrow cultures had much higher IGF-I mRNA levels compared to femoral cultures whether or not dex was present in primary culture. These findings demonstrate that dex supports the differentiation of both vertebral and femoral adult marrow osteogenic cells into osteoblasts. Our results support the hypothesis that osteoblastic marrow cultures differ depending upon which location in the skeleton they are from and that there are skeletal site–dependent differences in the insulin-like growth factor system components. J. Cell. Biochem. 71:382–391, 1998.

Key words: dexamethasone; bone marrow cell cultures; IGF-I; vertebrae

The loss of bone mass associated with aging and osteoporosis is due to an imbalance between bone resorption by osteoclasts and bone formation by osteoblasts. A thorough understanding of the factors that regulate osteoblast differentiation and activity should lead to the development of new approaches for preserving bone mass. Studies with fetal rat calvarial-derived osteoblast cultures have revealed an ordered sequence of gene and protein expression during osteoblast differentiation [Aronow et al., 1990; Owen et al., 1990; Stein and Lian, 1993]. Under specific culture conditions, cells from the calvaria multilayer to develop discrete areas termed nodules which mineralize. This in vitro model thus far has been useful for determining the factors that modulate the development of the osteoblast phenotype and bone formation. For example, the addition of glucocorticoid hormones increases the number and size of bone nodules formed in culture. The synthetic glucocorticoid dexamethasone (dex) selectively stimulates proliferation of the osteoprogenitor cells [Bellows et al., 1987, 1990] and promotes the differentiation of the osteoblasts in culture by initiating the expression of bone-related genes [Shalhoub et al., 1992, 1998].

In vitro models have also been designed which define bone cell differentiation and function in adult animals. A continuous source of osteoblasts is required for the purpose of new bone formation in the adult. These osteoblasts originate from pluripotent stromal stem cells within the bone marrow [Owen and Friedenstein, 1988; Beresford, 1989]. Adult rat stromal cell cul-

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tures derived from femur bone marrow form nodules which mineralize when dex is included in the culture medium [Maniatopoulos et al., 1988]. Analysis of gene expression in femoral bone marrow cultures reveals a pattern consistent with the osteoblast phenotype, and the presence of dex appears to be essential for maintaining this gene expression [Leboy et al., 1991; Kasugai et al., 1991; Rickard et al., 1994]. It remains to be shown if the numerous factors thus far identified in fetal and neonatal cultures as modulators of osteoblast development also represent the osteoblast biology of the adult.

With in vitro models of osteoblast development of adult-derived cells, there is the ability to define and compare the properties of marrow stem cells isolated from different anatomical sites [Sharrock, 1998]. Clinical and experimental data support the hypothesis that different locations within the adult skeleton respond differently to hormonal and therapeutic treatment and differ in the degree of bone loss associated with metabolic disease. For example, thyroid hormone (T_3) is necessary for normal skeletal development and maintenance, but excessive T_3 differentially decreases bone mineral density at the hip more than the spine [Diamond et al., 1991; Ongphiphadhanakul et al., 1993; Suwanwalaikorn et al., 1996]. Recently, we described a culture system that allows marrow cells isolated separately from adult rat femurs and vertebrae to both display osteoblast characteristics when cultured under identical conditions with dex present in the culture medium. Importantly, skeletal site–dependent differences are found with the in vitro addition of T_3, and the insulin-like growth factor I (IGF-I) transcripts are regulated differently in cells derived from vertebrae as compared to those from femurs [Milne et al., 1998].

Because it was known that femur-derived adult marrow cell cultures do not display osteoblastic properties when dex is omitted, our studies with vertebral-derived marrow cells also included dex in both primary and secondary culture. However, others have shown that glucocorticoids inhibit expression of IGF-I in fetal osteoblast and pre-osteoblast cultures [McCarthy et al., 1990; Delany and Canalis, 1995]. Glucocorticoids and insulin-like growth factors each play important roles in osteoblast function. No previous studies have examined the effects of dex on osteoblast differentiation in vertebrae-derived marrow cultures or the induction of IGF-I transcripts in the presence and absence of dex. In this study, we simultaneously compared the response to dex in cultures of young adult rat femoral and vertebral marrow cells. The cultures were examined for the development of the osteoblast phenotype markers including high alkaline phosphatase (AP) enzyme activity, and expression of mRNA for the bone matrix protein osteocalcin (OC). IGF-I gene expression was also examined. Our findings demonstrate that, like femur marrow cultures, maximal osteoblast differentiation in vertebral cultures does not occur when the cells are maintained in the absence of dex. However, in the presence or absence of dex, Northern analysis of cell cultures from both skeletal sites detects IGF-I gene transcripts, with gene expression being much higher in vertebral cultures than femoral cultures.

MATERIALS AND METHODS

All reagents were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise stated. The experimental protocol for animal use was in accordance with NIH Guidelines for the care and use of laboratory animals.

Cell Culture

We examined the influence of continuous exposure to dex on the osteogenic differentiation of femoral and vertebral bone marrow cultures. There were 12 experimental groups: six femoral culture groups and six vertebral culture groups. Femoral or vertebral cells in primary culture were maintained in either a medium with dex (10^{-8} M) or a medium containing vehicle (0.001% ethanol). In secondary culture, the vehicle and dex-treated groups were each subdivided into three groups: one with medium containing vehicle (control), one with medium containing dex at 10^{-8} M, and one with medium containing dex at 10^{-7} M.

Sprague-Dawley female rats, 100–125 g, were used as the source of bone marrow cells. Cultures were established as described previously [Milne et al., 1998], with some modifications. Briefly, cell suspensions were prepared separately from the vertebral and femoral bone marrow. Total, unfractioned bone marrow cells were seeded (day 0) at 5×10^6 cells/ml (9×10^5 cells/cm^2) on uncoated 100 mm plates (Corning, Cambridge, MA). The culture medium consisted of MEM supplemented with 20% heat-inactivated fetal bovine serum (FBS) (lot 7008C;

Atlanta Biologicals, Norcross, GA) and antibiotics. After 24 h (day 1), ascorbic acid (50 μg/ml) was added to all cultures, and treatment with either vehicle or dex (10^{-8} M) was started. Cells were allowed to attach for 72 h (day 3), at which time the medium was replaced with fresh medium containing MEM, 20% FBS, ascorbic acid, and either vehicle or dex. Culture medium was replaced again on day 6, and cell passage was performed on day 7, when femoral and vertebral cultures were 70–90% confluent. Secondary cultures were established by seeding trypsinized cells at 5.0×10^4 cells/ml (1.25×10^4 cells/cm^2) into six-well culture plates. Dex was present in the culture medium (none = control, 10^{-8} M, or 10^{-7} M) along with 20% FBS and ascorbic acid. The medium was replaced every 2 days thereafter, and cells were maintained for a total of 6 days after passage. All experimental groups were harvested on day 6 for either AP assays or gene expression studies.

Alkaline Phosphatase Histochemistry

Cultures grown in six-well plates were washed with cold phosphate-buffered saline (PBS), pH 7.4, and fixed with cold 2% paraformaldehyde in 0.1 M cacodylic buffer, pH 7.4. The fixed cultures were assayed histochemically for AP enzyme activity as described previously by Shalhoub et al. [1992]. Enzyme reactions for all experimental groups were incubated for 30 min. AP-positive staining in control and dex-treated femoral and vertebral cultures was documented by photographing macroscopically. All treatment groups were examined in duplicate wells. Quantification of the extent of AP-positive staining was done by first scanning the photographic images with a Microtek scanner (Redondo Beach, CA) and then analyzing the optical density of each well with Biosoft Quantiscan Software (Cambridge, England).

Northern Analysis

Total RNA from control and dex-treated femoral and vertebral marrow cultures was isolated from duplicate wells by the guanidinium thiocyanate method [Chomczynski and Sacchi, 1987]. The RNA (5 μg total RNA) was size-fractioned on 1.2% agarose/1.8% formaldehyde gels and Northern blot transfer performed on Nytran membranes (Schleicher and Schuell, Keene, NH). RNA was UV cross-linked to the membranes, and the membranes were hybridized with probes labeled with [^{32}P]dCTP (Amersham; Arlington Heights, IL) by the random primer method [Feinberg and Vogelstein, 1983]. The rat osteocalcin gene probe was a gel-purified insert from a plasmid kindly provided by Dr. Jane Lian [Lian et al., 1989] (University of Massachusetts Medical School, Worcester, MA). The insert used as the gene probe for rat IGF-I [Murphy et al., 1987] was provided by Dr. Ernesto Canalis (St. Francis Hospital and Medical Center, Hartford, CT).

The OC blots were exposed to BioMax MS film with a BioMax TranScreen-LE (Eastman Kodak, Rochester, NY) for 24 h at −80C. The IGF-I blots were exposed to MS film with intensifying screen for 108 h at −80C. The autoradiograms were quantified by scanning densitometry using Quantiscan Software. All mRNA levels were normalized to the amount of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA on the membranes as determined by hybridization to a GAPDH gene probe [Tso et al., 1985].

RESULTS

In marrow cultures from both femur and vertebrae, AP-positive colonies were first observed on day 3 of secondary culture and reached a maximum on day 18. For the present study, a preliminary time course was performed to determine the appropriate time point at which to simultaneously compare AP activity and osteoblastic gene expression of all experimental groups. It was observed that, while being fed medium on day 6 of secondary culture, those marrow cells derived from femurs and maintained in either 10^{-8} M dex or in vehicle (no dex) began to peel away from the culture plates (compare photographs, Fig. 1). Therefore, all determinations were performed on day 6 cultures in order to harvest samples of viable cells.

Dex Stimulates AP Activity of Femoral and Vertebral Marrow Cultures

Figure 1 shows that, when compared simultaneously and under identical culture conditions, femoral and vertebral marrow cells displayed differences in the extent of AP enzyme activity associated with the cell layers. Under all experimental conditions, femoral cultures expressed higher enzyme activity levels than vertebral cultures. The effects of varying concentrations

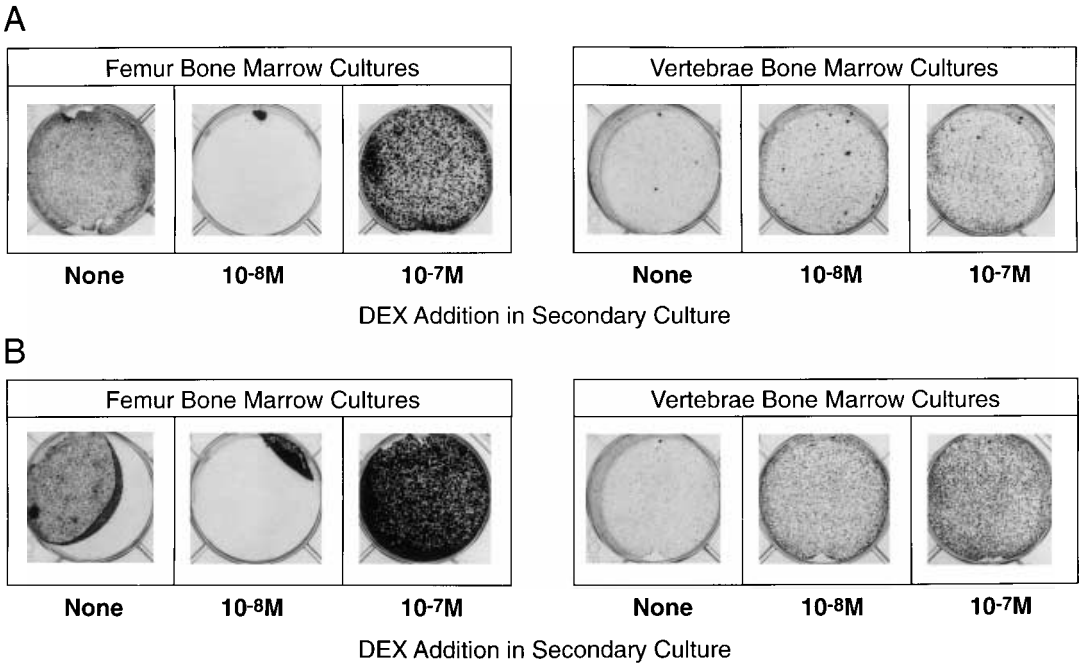

Fig. 1. Effect of dexamethasone addition on alkaline phosphatase activity of cultured rat femoral and vertebral bone marrow cells. **A:** Secondary cultures of cells without dex in primary culture. **B:** Secondary cultures of cells with dex in primary culture. Marrow cells from adult female femoral and vertebral bones were maintained in primary culture in MEM, 20% FBS, ascorbic acid in the absence (A) or presence (B) or dex (10^{-8} M). At day 7 of primary culture, the cells were trypsinized and replated at a density of 1×10^5 cells/35 mm well and maintained in secondary culture in medium with no added dex, 10^{-8} M dex, or 10^{-7} M dex. At day 6 of secondary culture, cells were fixed and stained histochemically for AP activity. Shown are representative photographs from duplicate wells for each experimental condition.

of dex on AP activity in secondary culture are also shown in Figure 1.

A stimulatory effect of dex added in secondary culture was seen whether the hormone was omitted in primary culture (Fig. 1A) or added at 10^{-8} M in primary culture (Fig. 1B). However, even though the cultures were AP-positive, femoral marrow cultures completely detached from the culture dishes by day 6 when maintained in 10^{-8} M dex in secondary culture and also began to detach when dex was omitted in secondary culture. A summary of these findings is presented in Figure 2, with all values expressed relative to the highest enzyme activity quantified in the culture wells.

Dex Increases OC mRNA Steady-State Levels in Femoral and Vertebral Marrow Cultures

Northern analysis performed on RNA isolated from both femoral and vertebral marrow cell secondary cultures on day 6 revealed that OC gene expression was not detected when dex was omitted in passaged cells even if dex had been present in primary culture (Fig. 3). Under all experimental conditions, OC gene expression was higher in vertebral cultures than in femoral cultures. Also, in both femoral and vertebral dex-treated secondary cultures, a concentration of 10^{-7} M induced OC expression substantially over a concentration of 10^{-8} M. The relative OC mRNA concentration on the autoradiograms as analyzed by densitometry is shown in Figure 4.

Vertebral Marrow Cell Cultures Have Higher IGF-I mRNA Steady-State Levels Than Femoral Marrow Cultures

Figure 5 shows that IGF-I gene expression could be detected in both femoral and vertebral cultures examined on day 6 of secondary culture. However, IGF-I mRNA levels were much greater in vertebrae-derived cells compared to femur-derived cells whether or not dex was present in primary culture. In femoral secondary cultures of cells without dex in primary culture, dex at 10^{-8} M and 10^{-7} M (in secondary culture) increased IGF-I expression compared with no dex. When dex was present in primary

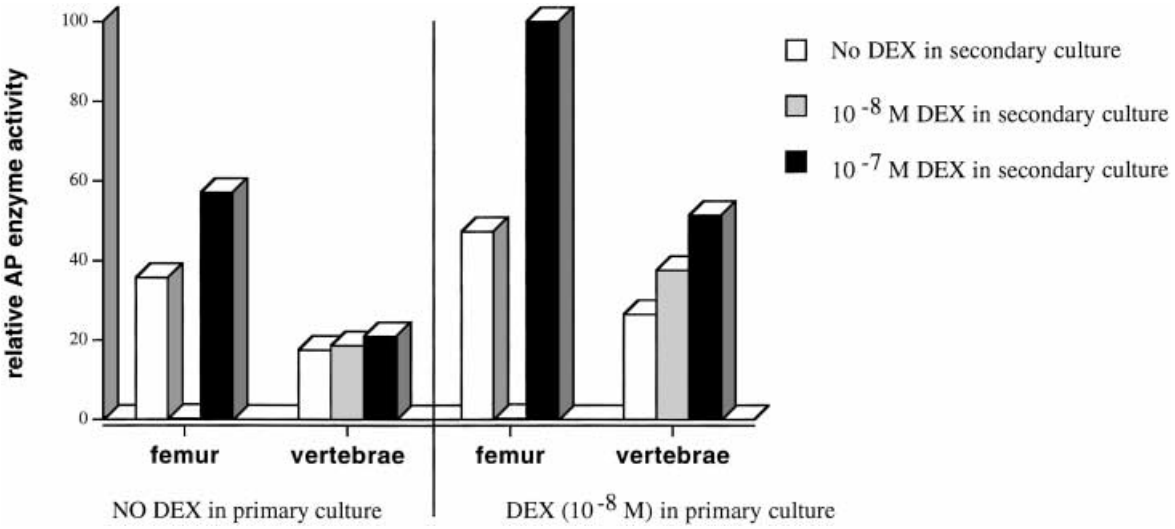

Fig. 2. Dose-dependency of the effect of dex on AP enzyme activity of femoral and vertebral bone marrow cultures. The AP activity of the histochemically assayed cultures (shown in Fig. 1) was quantified by measuring the optical density of each AP-positive culture well. The highest measurement was assigned a value of 100. The average relative value of AP activity of duplicate wells for each experimental condition is shown.

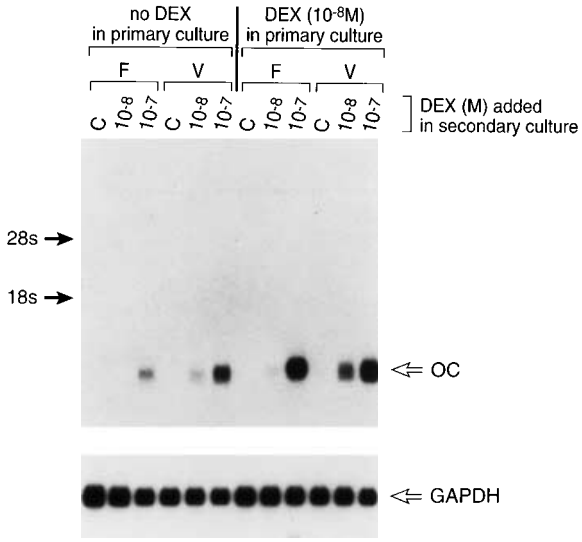

Fig. 3. Osteocalcin gene expression in femoral and vertebral bone marrow cultures. Femur (F) and vertebrae (V) marrow cells were grown in primary culture in the absence or presence of dex (10^{-8} M) and then passaged and maintained in secondary culture in vehicle (C) or 10^{-8} M or 10^{-7} M dex. Total RNA was extracted on day 6, and 5 µg/lane were used for Northern blot hybridization. The membrane was first probed with a [^{32}P]-labeled rat OC cDNA, which hybridizes to a 0.6 kb transcript. The membrane was also probed for the housekeeper gene GAPDH to assess sample loading. The positions of 28s and 18s ribosomal RNAs are indicated.

culture, dex did not further increase expression in secondary femoral cultures. In vertebral cultures, the presence of dex at 10^{-8} M and 10^{-7} M increased gene expression in secondary cultures of vertebral cells derived from primary cultures with or without dex. The relative IGF-I mRNA concentration on the autoradiograms as analyzed by densitometry is shown in Figure 6.

DISCUSSION

In the present study, we compared the expression of the osteoblast phenotype in femoral vs. vertebral rat bone marrow cell cultures in the absence and presence of the synthetic glucocorticoid dex. Also, IGF-I gene expression was compared in these cultures. Numerous studies on the differentiation of osteogenic cells in vitro underscore the importance of glucocorticoid supplementation to achieve maximal expression of osteoblast-related genes, but no previous studies have compared the responses to dex in osteoblasts cultured from marrow of different skeletal sites.

Previously we reported that bone marrow cells isolated simultaneously from adult rat femurs and vertebrae, when cultured separately under conditions known to induce osteogenic differentiation, both formed mineralized nodules in primary and secondary culture.

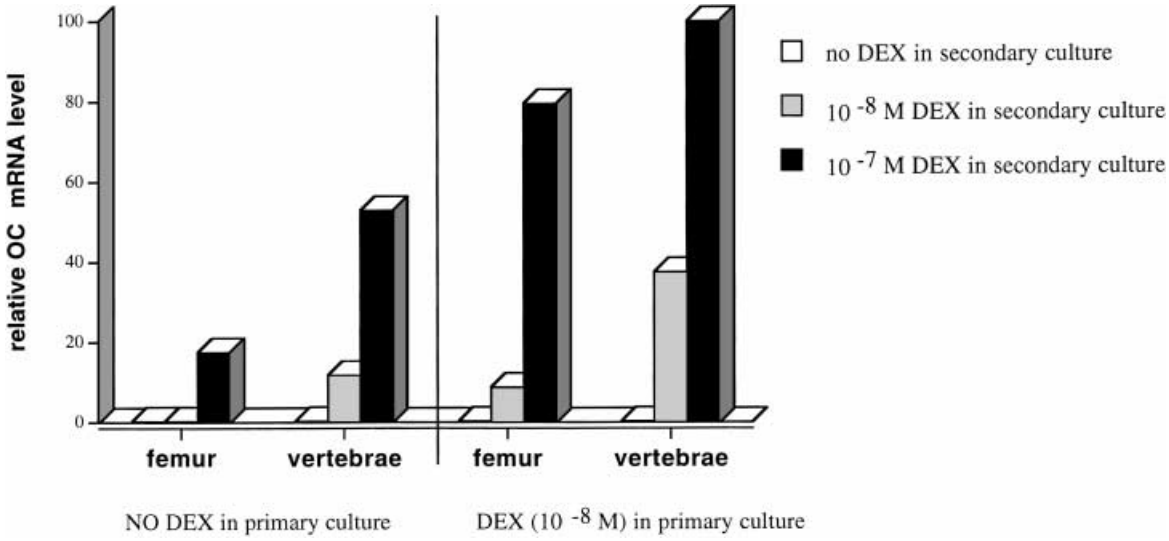

Fig. 4. Densitometric analysis of the Northern blot hybridizations for OC. The data are expressed as the relative OC mRNA concentration on the autoradiograms as analyzed by densitometry and normalized to the GAPDH mRNA level. The highest calculated OC/GAPDH ratio was assigned a value of 100. Values represent the mean of two separate experiments including the one shown in Fig. 3.

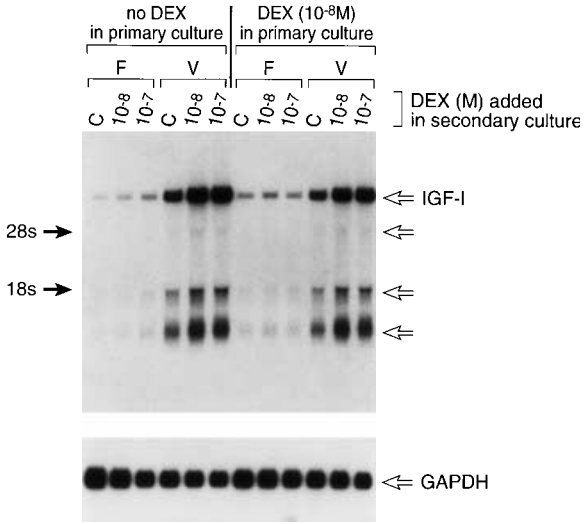

Fig. 5. Insulin-like growth factor I gene expression in femoral and vertebral bone marrow cultures. Conditions are described in Fig. 3. The sizes of the four transcripts detected were 6.5, 4.1, 1.7, and 0.9 kb.

Analysis of gene expression for the osteoblast markers OC and collagen type I indicated that, like the femur-derived marrow cultures, the vertebrae-derived marrow cultures displayed a temporal pattern of mRNA expression consistent with the development of the osteoblast phenotype [Milne et al., 1998]. For those studies, dex was included in the culture medium at a concentration of 10^{-8} M for primary culture and at 10^{-7} M for secondary culture.

Recently it was reported by Malaval et al. [1998] that dex added to fetal rat calvaria cultures downregulates the expression of leukemia inhibitory factor (LIF), a cytokine that inhibits osteoprogenitor differentiation and the expression of osteoblast-associated genes [Malaval et al., 1995]. It was proposed that this action of dex may in part explain how glucocorticoids promote bone formation in culture [Malaval et al., 1998]. Glucocorticoids also induce osteogenic cells in adult femoral marrow cell populations to differentiate in vitro. The cells express the enzyme alkaline phosphatase and the bone matrix proteins collagen type I, osteocalcin, bone sialoprotein, osteopontin, and osteonectin, and they form and mineralize bone nodules [Maniatopoulos et al., 1988; Leboy et al., 1991; Yao et al., 1994; Malaval et al., 1995; Rickard et al., 1994, 1995]. The osteogenic cells harvested from the adult marrow are in an earlier differentiation stage than those derived from calvaria. The marrow cell population established in culture consists of fibroblastic, adipocytic, and immunohematopoietic cells in addition to the osteoblast stem cells. It has been shown that dex supplementation in vitro alters

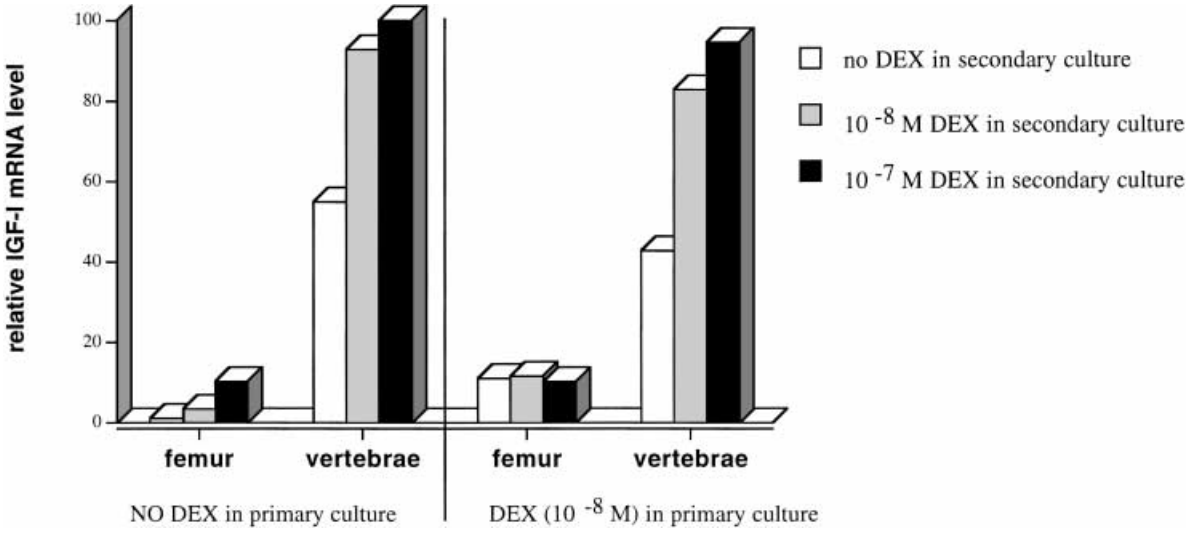

Fig. 6. Densitometric analysis of the Northern blot hybridization for IGF-I. Data are expressed as the mean of the ratio of the 6.5 kb IGF-I mRNA/GAPDH mRNA from two separate experiments including the one shown in Fig. 5. The highest calculated IGF-I/GAPDH ratio was assigned a value of 100.

this subpopulation makeup while stimulating osteoprogenitor differentiation [Herbertson and Aubin, 1995]. These accessory cell types seeded in culture along with the osteoblast stem cells play a role in supporting osteogenesis. Rickard et al. [1995] showed that nonadherent marrow cells, or the conditioned media derived from these cells, support the development of stromal cells into differentiated osteoblasts. Therefore, the adult marrow stromal cell culture model is useful for examining how factors such as therapeutic agents, disease status, age, and gender affect the osteoblast phenotype and bone formation. It is now recognized that the differentiation and function of osteoblasts, osteoclasts, stromal cells, and immunohematopoietic cells are linked to each other through a complex network involving cell-secreted factors and cell-cell contact. Thus, a marrow culture system where cells are not separated or sorted based on stage of differentiation or cell type could serve to identify the additional factors modulating bone formation [Sharrock, 1998].

Observations in patients and mature animals emphasize that different anatomic sites in the skeleton respond differently to hormones and therapeutic treatment. For example, in women with high turnover osteoporosis, treatment with calcitonin results in an increase in vertebral bone mineral density (BMD) but not femoral BMD [Civitelli et al., 1988]. In the rat, calcitonin increases vertebral but not femoral bone density [Ongphiphadhanakul et al., 1992], and this skeletal heterogeneity is reflected by specific changes in bone cell gene expression [Jenis et al., 1994]. In women with thyroid carcinoma, the high doses of thyroid hormone required to suppress thyroid-stimulating hormone release also cause a greater loss of bone mass at the hip than the spine. In adult rats, femoral and vertebral bones are differentially affected by long-term excessive thyroid hormone administration [Suwanwalaikorn et al., 1996]. These in vivo observations of skeletal site heterogeneity are supported by in vitro studies. Heersche et al. [1993] have shown that formation of mineralized bone nodules by cells derived from calvaria of young adult rats is more than tenfold greater than bone nodule formation by vertebral cells, femoral cortical bone cells, and femoral trabecular bone cells. In addition, these cell populations respond differently to progesterone in terms of mineralized bone nodule formation.

Given the abundant evidence of skeletal site–dependent differences in bone cell responsiveness, we sought to characterize a reproducible in vitro model to simultaneously compare the regulation of osteoblasts derived from the hip and spine, the two skeletal sites most adversely affected by bone loss due to aging and postmenopausal osteoporosis. As in cultures of adult femur bone marrow, we show that glucocorticoids such as dex are necessary for the expression of

the osteoblast phenotype in bone marrow cultures derived from adult rat vertebrae. In parallel with observations made in femur-derived marrow cultures, AP enzyme activity was greatly diminished if dex was absent in passaged vertebral cultures. OC gene expression in vertebral and femoral secondary cultures also required the presence of dex. However, the same was not true for IGF-I gene expression. Although the presence of dex did alter steady-state IGF-I RNA levels in cultures from both skeletal sites, gene expression was still evident in the absence of dex in primary and/or secondary cultures. Under all experimental conditions, IGF-I expression was higher in vertebral cells than in femoral cells.

Studies of the developmental control of osteoblasts in culture have shown that OC gene expression marks the presence of differentiated osteoblasts [Stein and Lian, 1993]. Based on our results regarding OC gene expression and AP activity, we conclude that the addition of 10^{-8} M dex to primary cultures, followed by 10^{-7} M dex for secondary cultures, supports maximal development of the osteoblast phenotype in both vertebral and femoral marrow cultures. We previously reported that these conditions also support extensive mineralization of cultures from both skeletal locations [Milne et al., 1998]. The present data regarding the IGF-I gene confirm our earlier findings that vertebral marrow cultures have higher expression than femoral cultures. Here we have extended those studies and demonstrate that this differential IGF-I gene expression is apparent in marrow cultures not exposed to dex. The reason for the skeletal site difference in IGF-I transcript levels can only be speculated at this time. OC gene expression was undetectable and AP activity was low when dex was not present in vertebral or femoral secondary cultures. Thus, it is unlikely that vertebral marrow cultures expressed higher IGF-I levels than femoral cultures simply due to the fact they were more representative of mature osteoblasts. In addition to osteoblasts, other cells present in femoral and vertebral cultures are contributing to the IGF-I gene expression in culture. Marrow stromal fibroblast-like cells, as well as differentiated osteoblasts, synthesize IGF-I [Zhang et al., 1991, 1995]. It is also possible that the properties of the osteoprogenitors and osteoblasts themselves are different because of varying extrinsic factors at the skeletal location the cells reside in, such that they have different potentials for proliferation and differentiation. Further studies investigating the mechanisms of this heterogeneity are in progress.

We have described a method where marrow cells were harvested from the bones of separate skeletal locations, expanded in primary culture, passaged, and further maintained under the same culture conditions for 6 more days. Yet, even in this identical environment, we still observed clear differences between femoral and vertebral osteogenic cultures. This suggests that factors intrinsic to the cells from each skeletal site continue to be expressed and direct cell differentiation in culture. In other words, the marrow cells retain a memory of their original location, so it may therefore be possible to identify and examine these factors. This ability of cultured osteogenic cells in vitro to retain a memory of their in vivo origins was also observed by Ishida and Heersche [1997]. They reported that, in cells derived from rat lumbar vertebrae explant cultures, the steroid hormone progesterone increased bone nodule formation in female-derived populations but not male-derived populations.

It is of clinical relevance to define and compare the properties of osteoblasts from different anatomic sites, and numerous in vitro models of human osteoblast differentiation have been described [Beresford et al., 1993; Cheng et al., 1994]. However, seldom is it feasible to simultaneously compare osteoblast differentiation of marrow stem cells isolated from separate skeletal sites of the same human donor. In the animal model described here, femoral marrow cells are compared in parallel with vertebral marrow cells from the same animals. Consistent with previous studies demonstrating that glucocorticoids are essential for osteoblast differentiation in human marrow cultures [Cheng et al., 1994], we found that rat vertebral and femoral cultures both require dex. Also, our findings regarding vertebral vs. femoral IGF-I gene expression are consistent with results indicating that there are skeletal site–dependent differences in the production of IGF-I system components by normal human bone cells in culture [Malpe et al., 1997]. In summary, these data support the validity of using adult rat marrow osteogenic cell cultures to examine os-

teoblast differentiation and study the phenomenon of skeletal site heterogeneity.

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