Long-term culture in dexamethasone unmask an abnormal phenotype in osteoblasts isolated from osteoporotic subjects

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ABSTRACT. We have shown that osteoblastic cells derived from trabecular bone explants of osteoporotic subjects (OP cells) exhibited an altered alkaline phosphatase (ALP) response to 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] compared to control (CON) cells. Our hypothesis that OP cells have other intrinsic abnormalities was investigated using our cell models representing two different stages of differentiation. OP and CON cells were cultured in the absence (-DEX) or presence (+DEX) of 10 nM dexamethasone (DEX) in 10% fetal calf serum (FCS) prior to exposure to serum-free medium containing 1 nM of PTH and/or 17-β estradiol (E₂). Both OP and CON cells responded to DEX with a two-fold increase in basal ALP activity. While E₂ or PTH+E₂ had no effect on OP cells, both treatments inhibited ALP activity in CON cells (p<0.05). OP and CON cells grown in DEX also expressed PTH-stimulated adenylyl cyclase (AC) activities higher than those of (-DEX) cells. OP+DEX cells, however, exhibited activities which were 8-fold higher than those of CON+DEX cells (p<0.001). In OP+DEX cells, E₂ stimulated basal AC activity (p<0.05) but did not affect PTH-stimulated activity. In contrast, in CON+DEX cells, E₂ had no effect on basal activity but inhibited PTH-stimulated AC activity (p<0.001). Osteocalcin production was 4-fold lower in OP+DEX cells compared to OP-DEX and CON cells (p<0.05) while osteocalcin mRNA levels were significantly lower in OP+DEX and CON+DEX cells compared to OP-DEX cells (p<0.05). E₂ did not affect osteocalcin protein or mRNA levels in either OP or CON cells. No differences in mRNA levels were found for estrogen receptor-α (ER-α) in OP+DEX cells whereas these levels were significantly higher in CON+DEX compared to CON-DEX cells (p<0.05). These results indicate that DEX amplified the differences between OP and CON cells and confirm the presence of intrinsic osteoblastic abnormalities in patients with osteoporosis that persist in culture.


INTRODUCTION
Osteoporosis is a heterogeneous condition characterized by decreased bone mass and a propensity for fractures, occurring in 40% of women and 13% of men over the age of 50 (1-4). Factors including estrogen deficiency (1, 3) and age-related decreases in hormones that maintain calcium homeostasis (1) contribute to bone loss in post-menopausal women. In addition, genetic factors are currently receiving considerable research attention (3).

In post-menopausal osteoporosis, the major abnormality leading to a remodelling imbalance is generally thought to be an increase in bone resorption. On the other hand, histomorphometric studies have shown that a significant proportion of patients with idiopathic osteoporosis have reduced bone formation with decreased osteoblast activity (5, 6). Also, studies suggested that in aging rats, age-related bone loss is linked to a defect in osteoblast recruitment (7, 8). Our in vitro study of osteoblastic function in osteoporosis showed that osteoblastic cells cultured from trabecular bone explants of osteoporotic subjects (OP cells) had increased alkaline phosphatase (ALP). Responsiveness to treatment with 1,25-dihydroxyvitamin D₃ [1,25 (OH) 2D₃] compared to controls (9). This finding is convergent with those of Duda et al. (10), who found higher serum osteocalcin responsivity to 1,25-dihydroxyvitamin D₃ in osteoporotic women. We
therefore postulated intrinsic cellular abnormalities in osteoblasts of patients with osteoporosis (9).
In order to probe this hypothesis, we extended our studies on osteoporotic (OP) cells and compared additional osteoblastic properties with those of cells from age-matched, non-osteoporotic control subjects (CON cells), using our two stage osteoblastic model which we developed as a probe for differentiation-dependent events. This model takes advantage of two culture conditions under which human osteoblastic cells exhibit characteristics indicative of two different stages of differentiation (11-13). Cells continuously cultured in the presence of 10 nM dexamethasone (DEX) consistently had higher ALP and PTH-stimulated adenylyl cyclase activities compared to cells grown in the absence of DEX, suggesting a more differentiated phenotype. Using this model, we have compared the effects of 17β-estradiol (E2) and bovine PTH [bPTH (1-84)] on osteoblastic function in OP and CON cells.

MATERIALS AND METHODS

Subjects
Iliac crest trabecular bone biopsies were obtained from 19 non-osteoporotic (7 females, 12 males) and 12 osteoporotic (11 females, 1 male) subjects using a bone sampling protocol approved by the Human Studies Review Committee of the University of Toronto. Control subjects were undergoing surgery for orthopedic procedures necessitating iliac crest bone grafts, and for the most part were diagnosed as having degenerative disk disease. None had obvious features of metabolic bone disease or malignancy by history, physical examinations, biochemical or radiographic studies. Osteoporotic subjects were patients with idiopathic osteoporosis who were being treated at the Metabolic Bone Clinic, St. Michael's Hospital. The diagnosis of idiopathic osteoporosis was based on a bone mineral density of 2 SD below the normal mean and/or evidence of vertebral fractures (9), after excluding secondary causes. Serum calcium, PTH and 25-hydroxyvitamin D levels were within the normal range in all patients. There were no significant differences in the ages of the two groups of subjects. A summary of the demographic features of these subjects is shown in Table 1.

Cell cultures
The culture conditions were as described previously (9, 11-13). Briefly, bone fragments were cleaned of adherent tissue and blood, cut into 1 to 3 mm pieces, divided equally, and cultured separately in 75 cm² flasks containing media [Ham's F-12 supplemented with 28 mM HEPES, 1.1 mM CaCl₂, 1% glutamine, 1% antibiotic-antimyocotic solution and 10% fetal calf serum (FCS)] with or without 10 nM DEX. The normal physiological concentration of glucocorticoids (cortisol) ranges from 100 to 600 nM (MedlinePlus Encyclopaedia). In comparison, culture media containing FCS and to which DEX has not been added contains about 10% of this value. The medium was replaced after 24 h and twice weekly thereafter. After 4 to 6 weeks, by which time the cells had migrated from the bone and attained confluency, the cells were harvested by trypsinization and subcultured at a density of 1x10⁴ cells/cm² into 12-well dishes and/or 25 cm² flasks, depending on the number of cells available. All studies were performed with cells from the first passage. For E₂ studies, the medium was replaced on the eighth day with serum-free medium ± DEX. The following day, vehicle, 1 nM E₂ and/or PTH were added and the cells cultured for an additional 24 h.

RNA isolation
Cells were harvested from 25 cm² flasks by trypsinization and the total RNA isolated by the method of Chomczynski and Sacchi (14). Briefly, cells were lysed with acidic guanidine thiocyanate and the RNA isolated by phenol/chloroform extraction. The RNA was further purified by repeated ethanol precipitations.

Slot blot analyses
Because the limited numbers of cells available yielded insufficient RNA for Northern, the RNA samples were usually analysed by slot blots to quantify mRNA levels. Five micrograms of RNA, in duplicate, were applied onto nylon membranes (Amersham, Arlington Heights, IL) using a Tyler slot blot manifold (Bio/CAN, Mississauga, Ontario). Northern analyses were used to determine the specificity of the cDNA probes. The blots were hybridized with 32P-labelled CDN As for ALP (human, 2.5 kb) (15), collagen type I (COLL, porcine, 0.3 kb, 3'-untranslated region), osteocalcin (OC, rat, 0.6 kb), receptors for E₂ (human, 1 kb) (16) and PTH (human, 1.0 kb, PvuII-PvuII) (17) and glyceraldehyde-3-phosphate dehydrogenase (GAPD, mouse, 1.0 kb) for 2 h in Rapid Hybridization Buffer (Amersham) at 65 C prior to washing and exposure to X-ray film, according to the manufacturer's (Amersham) directions. All cDNAs hybridized to the mRNA species reported in the literature (15-19). mRNAs for the phenotypic markers were quantified by successive hybridizations of each slot blot. mRNA levels were expressed as densitometric ratios of each mRNA and GAPD mRNA.

ALP activity
At confluence, cells grown in 12-well dishes were washed twice with 50 mM Tris HCl, pH 7.3, harvested by scraping, and stored at −20 C until ready for assay. ALP activity was determined in cell sonicates as previously described (11, 20). ALP activity was expressed as nmol p-nitrophenol released from p-nitrophenyl phosphate/mg protein/min of incubation at 30 C.

Adenylyl cyclase activity
Adenylyl cyclase (AC) was assayed as described previously in cells that had been treated for 24 h with vehicle or E₂, using a modification of the method of Shimizu et al. (11, 21). Cells were