Age and gender specific stimulation of creatine kinase specific activity by gonadal steroids in human bone-derived cells in culture

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ABSTRACT. We previously reported a non-enzymatic method for isolation of human bone cells in culture that display osteoblastic features and respond to 1,25 dihydroxy vitamin D (1,25) and to parathyroid hormone (PTH). The present study was undertaken to analyze the response of cultured human bone cells to 17β-estradiol (E2) and to dihydrotestosterone (DHT) as a function of gender and age. Cultured human bone cells, obtained from biopsies during orthopedic surgery, were divided into four groups defined by gender and age: pre- and post-menopausal healthy non-osteoporotic women that were not under hormone replacement therapy (HRT) and mature (<55-year-old) and older (>60-year-old) men. We found gender specific responses to gonadal steroids using the specific activity of the brain type (BB) isozyme of creatine kinase (CK) as a response marker. Constitutive levels of CK activity did not change with age or gender and the enzyme extracted from cells from the different sexes and ages did not respond to either progesterone (P) or to 1,25. CK from the different cells responded to gonadal steroids in a gender specific manner, *i.e.* CK from female derived cells responded to E2 only and the enzyme from male derived cells responded to DHT only. In female derived cells the response to E2 declined significantly with age, while the response to DHT in CK from male derived cells did not vary with age. This may be due to either decreased proportion of mature osteoblasts and/or their differentiation state and/or changes in the levels of estrogen receptor(s), coactivators or corepressors in these cells. These results extend our knowledge of human osteoblast biology (beyond murine cells) and are therefore more relevant for developing models for treatment of human metabolic bone diseases such as post-menopausal osteoporosis.

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INTRODUCTION

Gonadal steroids play a crucial role in skeletal development and maturation (1-4). Estrogen depletion after menopause is associated with a reduction in bone mass and is recognized as a risk factor for osteoporosis and its resulting fractures. Estrogen replacement therapy can delay or prevent bone loss (1-4). Most of these deleterious changes are thought to be the result of low estrogen levels, but lack of progesterone (P) may also contribute (5). In human males, although there is no cessation of testicular function comparable with the menopause in women, both total and free testosterone levels decline with age (6) and a common secondary cause of osteoporosis in men is hypogonadism. It has recently been demonstrated that estrogens as well as androgens are also necessary for the maintenance of the male skeleton (6, 7).

The failure for many years to demonstrate either estrogen receptors in bone cells (8) or direct estrogen

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responsiveness of bone in organ or cell cultures (9, 10) led to the notion that estrogens exert their effects indirectly by mechanisms concerned mainly with decreased bone resorption. However, estrogen receptors and their mRNA transcripts have been found in cultured murine and human bone cells (11-13) as well as in human embryonic bone (13). Estrogen has also been shown to influence the proliferation and differentiation of rodent osteoblast-like cells and human osteosarcoma cell lines (14-16). Androgen receptors have been identified in normal human osteoblast-like cells (17) and it has been shown that androgens stimulate bone cells proliferation *in vitro* (18, 19). The presence of P receptors, as well as the effects of P on cell proliferation, have also been demonstrated in human osteoblast-like cell lines (5, 19-21).

The brain type (BB) isozyme of creatine kinase (CK) is an enzyme involved in the "energy buffer" system, which regulates cellular concentration of ATP and ADP. It is the major component of the "E2-induced protein" found in rat uterus and other tissues containing 17β-E2 (estradiol) receptors (22). CK stimulation is an efficient response marker to detect biological activity of E2 (22-24) particularly in the case of osteoblasts which contain low concentrations of E2 receptors (11, 12). The stimulation of CK in cultured bone cells requires the higher end of the physiological range of estrogen concentrations (19). CK is also a general marker for the action of hormones, which, like estrogens, cause increased utilization of energy for processes such as cell growth (22-25). Although it is not a specific marker for bone, it is a very useful marker for the activity of gonadal steroids and was shown to be correlated with markers of bone formation *in vivo* (26).

The stimulatory effects of gonadal steroids on bone are gender specific, since estrogens are active only in females and androgens only in males. This specificity was found in rat diaphyseal bone (18, 27) and in mice epiphyseal cartilage and diaphyseal bone (28), as well as in bone-derived cells such as rat embryo calvaria cells in culture (19). This was also true for human osteoblasts in culture such as SaOS-2 cell lines (29) and primary human bone cells in culture (30). Rat cartilage cells in culture (19) or rat cartilage *in vivo* (18) did not show any gender specific response to gonadal steroids although there are sex dependent effects of E2 on chondrocyte differentiation in culture (28, 29, 31).

Recently, we established a primary human bone-derived osteoblast-like cell culture system, exhibiting major osteoblastic characteristics, *i.e.* high level of basal alkaline phosphatase activity and responsiveness to 1,25 $(OH)_2D_3$ (1,25) and stimulated cAMP formation in response to PTH, and high levels of osteocalcin and its stimulated production by 1,25 (32).

In our model, only cells derived from female bone showed minor age-related changes. Higher basal activity of alkaline phosphatase and higher responsiveness to 1,25 as well as higher level of osteocalcin were found in pre-menopausal cells compared to post-menopausal cells. Osteoblastic cultures derived from male bones at similar age groups did not show any age dependent differences.

Using this culture system we analyzed whether these cells respond to gonadal steroids, using the stimulation of CK as a response marker, and whether these effects are gender and/or age dependent.

We present evidence that the effects of both estrogen and androgen on normal human cultured osteoblasts are gender specific and that the responses are age-dependent in females, but not in males in similar age groups.

MATERIALS AND METHODS

Cell isolation and culture

We used a modified procedure of Beresford *et al.* (33). Normal human bones were obtained from biopsies of patients undergoing corrective surgery following accidental injury or hip replacement. The experimental groups were divided into male and female. The male groups consisted of 17 healthy males: mature (under 55-year-old, no.=12) and older (over 60-year-old, no.=5). The female groups consisted of 23 healthy, non-osteoporotic females that were not receiving hormone replacement therapy (HRT): pre-menopausal (under 50-year-old, normally menstruating, no.=7) and post-menopausal (over 55-year-old, not menstruating, no.=16). The trabecular surface of the iliac crest or long bones were crushed into small (1 mm^3) pieces, then extensively and repeatedly washed with phosphate buffered saline (PBS) to remove blood components. The explants were seeded in 100 mm tissue culture dishes and incubated in DMEM medium without Ca^{++} containing 10% fetal calf serum (FCS) and antibiotics. Cell outgrowth from the trabecular bone surface was apparent after 6 days and the cells were then cultured until sub-confluence in phenol red free DMEM with 10% charcoal stripped FCS. Cells were seeded at a density of $3x10^5$ cells/35 mm tissue culture dishes.

Hormonal treatment

Sub-confluent cell cultures were incubated in triplicate for 24 h with increasing concentrations of E2, P, dihydrotestosterone (DHT) or vehicle (0.01% ethanol). At the end of incubation CK was extracted and assayed as described previously (18, 19).

Enzyme extraction and assay

At the end of incubation cells were washed with cold PBS, homogenized by freezing and thawing three times in cold extraction buffer (50 mM Tris NaCl, 5 mM magnesium acetate, 2.5 mM dithiothreitol, 0.4 mM EDTA, and 250 mM sucrose, pH 6.8). Homogenates were centrifuged at 14,000 g for 5 min at 4 C. The supernatant extracts were stored at -20 C. CK activity was measured at 340 nm in a Uvicon, Kontron automatic computerized recording spectrophotometer, using the Sigma coupled assay kit (18). A unit of enzyme activity was defined as the amount yielding 1 µmole ATP/min at 30 C and the specific activity as nmol/min/mg protein. Protein was assayed by the Coomassie blue method using BSA as standard (34).

Statistical analyses

Wilcoxon's signed-ranks test was performed to test the effect of the treatment within groups. Dose-dependent differences within the group were analyzed using Bonferroni test. Non-parametric Mann-Whitney U-test for two independent samples analyzed differences between age groups of each gender.

RESULTS

Constitutive CK activity in cultured bone cells

The basal activity of CK in cells derived from pre- and post-menopausal women showed no gender or age changes (22.6±3.25 and 20.8±3.7 nmol/min/mg protein, respectively). Similarly, male derived cells from similar age groups showed no significant difference in basal activity of CK between age groups of mature males compared to older males (28±6.5 and 24.6±4.0 nmol/min/mg protein, respectively) (Table 1).

Table 1 - *Age and gender effect on basal creatine kinase (CK) specific activity.*

Gender	Age	CK specific activity (nmol/min/mg protein)
Females	Pre-menopausal no.=7	22.6±3.25
	Post-menopausal no.=16	20.8±3.7
Males	Mature no.=12	28.0±6.5
	Older no.=5	24.6±4.0

Bone cells derived from pre- or post-menopausal women and mature or older men were cultured as explained in Methods and Materials. CK was extracted and assayed as described. The results are expressed as mean±SEM of the specific activity as nmol/min/mg protein.

Dose-dependent stimulation of CK by gonadal steroids in human cultured bone cells

Bone-derived cells obtained from females showed a dose dependent stimulation by E2 of CK specific activity with a maximum at 10 nM in both female groups (Fig. 1). Cells from pre-menopausal females responded even to a lower dose of E2 (1 nM). On the other hand, treatment with DHT caused no significant change in bone cells derived from females in any age group. Bone-derived cells obtained from males, showed a dose dependent stimulation by DHT of CK specific activity at 100 nM and 1000 nM in both age groups, but no stimulation at any age or concentration by E2 (Fig. 2; Table 2).

Maximal responsiveness of cultured bone cells: gender and age specific stimulation

The stimulations of CK activity by E2 in both cell types compared to untreated cells was significant

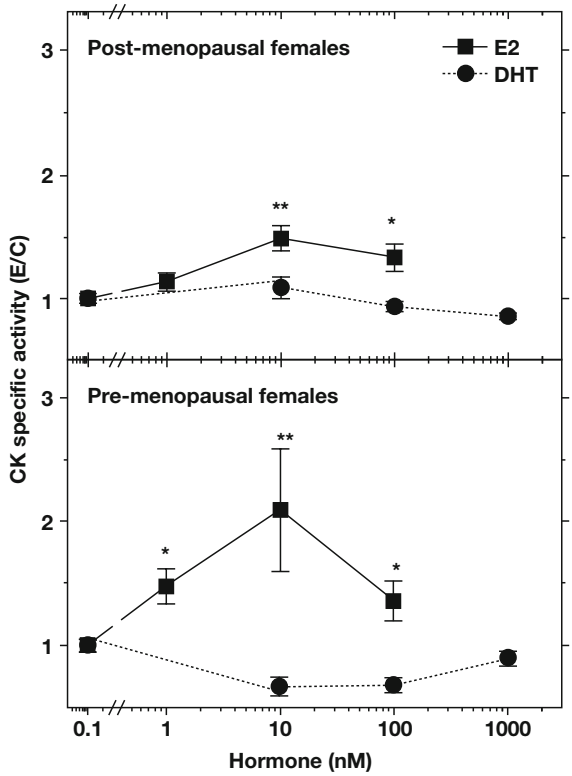

Fig. 1 - *Dose dependent stimulation of creatine kinase (CK) specific activity in cultured female human bone-derived cells. Bone cells derived from pre- and post-menopausal women (no.=7 and 16 respectively) were cultured and treated as explained in Materials and Methods. Cells were treated with estradiol (E2), dihydrotestosterone (DHT) or vehicle (C) for 24 h. CK was extracted and assayed as described. The results are expressed as means±SEM for experimental/control (E/C). Statistical analysis by Bonferroni test. *p=0.05, **p=0.01, **p=0.001.*

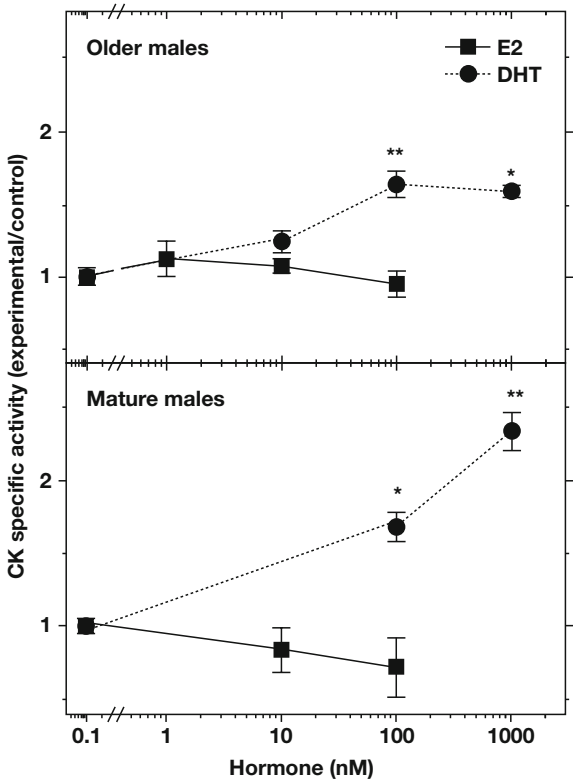

Fig. 2 - *Dose dependent stimulation of creatinine kinase (CK) specific activity in cultured male human bone-derived cells. Bone cells derived from mature and older males (no.=12 and 5 respectively) were cultured and treated as explained in Materials and Methods. Cells were treated with estradiol (E2), dihydrotestosterone (DHT) or vehicle (C) for 24 h. CK was extracted and assayed as described. The results are means±SEM for experimental/control (E/C). Statistical analysis by Bonferroni test. *p=0.05, **p=0.005.*

in both age groups (2.1 in pre- and 1.51 fold stimulation in post-menopausal cells, p=0.05 for both) (Fig. 1; Table 2). In cell cultures derived from post-menopausal female bones, E2-induced CK activity was significantly lower than in cells from pre-menopausal female bone (p=0.05) (Table 2).
While the stimulation of CK activity by DHT, in each male cell type, compared to untreated cells was significant in both age groups (p=0.05 in mature and p=0.001 in older cells at 100 nM) (Fig. 2; Table 2), no difference was found between cells derived from the two age groups (Table 2). Cells derived from both males and females showed no significant stimulation of their CK activity by either P (1 μM) or 1,25 (100 nM) (Table 2).

DISCUSSION

Our cell culture system of osteoblasts, derived from human bone explants, expressed osteoblastic features such as stimulation of cAMP formation by PTH, high levels of alkaline phosphatase activity and induction by 1,25$(OH)_2D_3$ and osteocalcin synthesis and its induction by 1,25$(OH)_2D_3$ (32). Human osteoblasts (33), human embryonic bone and cartilage (19) express receptors for gonadal steroids. Indeed, estrogens and androgens are active in our cell cultures in a gender specific manner (Figs. 1 and 2), *i.e.* female-derived cells respond only to E2 and male-derived cells only to DHT, using the specific activity of CK (BB CK) as a response marker to gonadal steroids (24). Although this marker is not a specific bone marker, it is one of the most sensitive markers for gonadal steroid action (22) and it was also correlated *in vivo* with calciotropic hormone activity and increased bone formation markers (26). This gender specificity was shown previously for rat calvaria cells in culture (18, 19), for primary human bone cells in culture (32) and for the human osteoblasts-like cell line $SaOS_2$ (29). This specific response was abolished upon gonadectomy and was retained upon treatment with the hormones, probably via changes in the concentrations of estrogen receptors (ERs) or their sub-types (27).

Table 2 - *Maximal induction of creatine kinase by gonadal hormones. Results are experimental/control (E/C).*

Treatment	Females		Males	
	Pre-menopausal	Post-menopausal	Mature	Older
E2 (30 nM)	2.1±0.50**	1.5±0.10**	0.7±0.23	1.05±0.07
DHT (300 nM)	0.5±0.10	0.65±0.08	1.67±0.10**	1.63±0.10*
P (1 μM)	1.1±0.20	1.07±0.05	1.47±0.30	1.2±0.25
1,25 (10 nM)	1.12±0.16	0.97±0.08	1.3±0.26	1.2±0.06

Bone cells derived from pre- or post-menopausal women and mature or older men were cultured and hormonally treated as explained in Methods and Materials. CK was extracted and assayed as described. Results are expressed as the ratio of hormonally treated compared to vehicle control treated; E/C for no.=3-16. Basal CK activities: pre-menopausal women=24±6.7, post-menopausal women=24.5±4.0, mature males=32.6±6.1, older males=26.1±5.2 nmol/min/mg protein. The results are expressed as mean of E/C±SEM. Statistical analysis by Bonferroni test. *p=0.05, **p=0.01).

Although the constitutive level of CK was similar in bone cells derived from both pre- and post-menopausal women, the response of pre-menopausal cells to E2 was 50% higher than that of post-menopausal cells ($p<0.005$) (Fig. 1) (Table 2). This may indicate that under these culture conditions, the growth of the cells from different origins is similar, but the content of receptors to the gonadal steroids and/or the co-activators and/or the co-repressors are different, and are modulated in female bones with age. This extent of CK stimulation paralleled the constitutive synthesis of osteocalcin, a parameter of bone differentiation, which declined in cells derived from bones of post-menopausal women (32). Our bone samples were obtained from pre-menopausal and post-menopausal women without osteoporosis in their medical history. The post-menopausal cell populations, showing a decreased response, could have less functional osteoblasts, or they might have lost some of their ability to respond to E2 by either a decrease in the number of receptors or co-receptors and/or other factors. However, Ankrom *et al.* (35) found in human post-menopausal women osteoblasts strains a greater concentration of ERα along with a reduction in their induction of hydroxyproline by estrogen. They interpreted this as a loss of receptor regulation and diminution of ligand-receptor signal transduction with increased age. Our results of age-associated decrease of CK-response to E2 can support their conclusions, provided that changes in ERβ are also measured, since they are important in bone (36). Whether this is due to decreased number of receptors or decreased functionality has to be further investigated.

We have previously demonstrated that gonadectomy of rats or of mice leads to the loss of gender specific response in rat bone to gonadal steroids and in both cartilage and bone in mice (27, 28). This can be restored by replacement with the steroid hormones (27). However, bone cells from post-menopausal women in culture, which might be equivalent to bone cells from ovariectomized rats or mice, did not lose their sex specific response and did not acquire responsiveness to DHT (Fig. 1), even if their response to E2 was significantly reduced. One possibility for the difference between species could be the relative loss of circulating estrogen in post-menopausal women, compared to post-ovariectomy in rats or mice, and/or in the threshold levels of the sex steroids needed for induction of CK, although there is no evidence for this since serum estrogen levels sharply decreased after menopause in both types. Circulating E2 in post-menopausal women (0-20 pg/ml) declines four orders of magnitude compared to the levels in pre-menopausal women (40-80 pg/ml) (37). The same magnitude of decline was observed in ovariectomized rats: 3.8 *vs* 13.7 pg/ml in sham rats (37, 38). Also, the same concentrations of E2 are needed for the induction of CK activity in humans and rats derived osteoblasts in culture (38).

The results presented in this study extend our knowledge of human osteoblast biology (beyond murine cells) and are therefore more relevant for developing models for treatment of human metabolic bone diseases such as post-menopausal osteoporosis.

REFERENCES

1. Christiansen C.
   Hormonal prevention and treatment of osteoporosis. State of art.
   J. Steroid Biochem. Mol. Biol. 1991, *6:* 1124-1130.
2. Gallagher J.C.
   The pathogenesis of osteoporosis.
   Bone Miner. 1990, *9:* 215-227.
3. Lindsay R.
   Sex steroids in the pathogenesis and prevention of osteoporosis.
   In: Riggs B.L., Melton III L.J. (Eds.), Osteoporosis: Etiology, diagnosis, and management.
   Raven Press, New-York, 1988, p. 333.
4. Peck W.A.
   Osteoporosis and estrogen deprivation. Experimental approaches to therapy: an overview.
   Postgrad. Med. 1987, *253:* 33-34.
5. Lukert B.P., Johnson B.E., Robison R.G.
   Esterogen and progesterone replacement therapy reduces glucocorticoid-induced bone loss.
   Bone Miner. 1992, *7:* 1063-1069.
6. Ebeling P.P.
   Osteoporosis in men. New insights into aetiology, pathogenesis, prevention and management.
   Drugs Aging 1998, *13:* 421-434.
7. Smith E.P., Boyd J., Frank G.R., Takahashi H., Cohen R.M., Specker B., Williams T.C., Lubahn D.B., Korach K.S.
   Estrogen resistance caused by a mutation in the estrogen receptor gene in a man.
   N. Engl. J. Med. 1994, *331:* 1056-1061.
8. Chen T.L., Feldman D.
   Distinction between alpha-fetoprotein and intracellular estrogen receptors: evidence against the presence of estradiol receptors in rat bone.
   Endocrinology 1978, *102:* 236-244.
9. Caputo C.B., Meadow D., Raisz L.G.
   Failure of estrogens and androgens to inhibit bone resorption in tissue culture.
   Endocrinology 1976, *98:* 1065-1068.

10. Van Passen H.C., Poortman J., Borgat-Creutzburg I. H.C., Thijssen J.H.H., Duursman S.A.
Oestrogen binding proteins in bone cell cytosol.
Calcif. Tissue Res. 1978, *25:* 249-254.

11. Komm B.S., Terpening C.M., Benz D.J., Graeme K. A., Gallegos A., Korc M., Greene G.L., O'Malley B.W., Haussler M.R.
Estrogen binding, receptor mRNA and biological response in osteoblast-like osteosarcoma cells.
Science 1998, *241:* 81-84.

12. Eriksen E.F., Colard D.S., Berg N.J., Graham M.L., Mann K.G., Spelberg T.C., Riggs B.L.
Evidence of estrogen receptors in normal human osteoblast-like cells.
Science 1988, *241:* 84-86.

13. Ben-Hur H., Mor G., Blikstein I., Likhuman I., Kohen F., Dgani R., Insler V., Yaffe P., Ornoy A.
Localization of estrogen receptors in long bones and vertebrae of human fetuses.
Calcif. Tissue Int. 1993, *53:* 91-96.

14. Ernst M., Schmid C., Froesch E.R.
Enhanced osteoblast proliferation and collagen gene expression by estradiol.
Proc. Natl. Acad. Sci. USA 1988, *8:* 2307-2310.

15. Gray T.K., Flynn T.C., Gray K.M., Nabell L.M.
17β estradiol acts directly on the clonal osteoblast cell line UMR 106.
Proc. Natl. Acad. Sci. USA 1987, *84:* 854-857.

16. Schevven B.A.A., Damen C.A., Hamilton N.J., Herhaar H.J.J., Duursma S.A.
Stimulatory effects of estrogen and progesterone on proliferation and differentiation on normal human osteoblastic-like cells in vitro.
Biochem. Biophys. Res. Commun. 1992, *186:* 54-60.

17. Colvard D.S., Eriksen E.F., Keeting P.E., Wilson E.M., Lubahn D.B., French F.S., Riggs B.L., Spelberf T.D.
Identification of androgens receptors in normal human osteoblast-like cells.
Proc. Natl. Acad. Sci. USA 1989, *86:* 854-857.

18. Sömjen D., Weisman Y., Harell A., Berger E., Kaye A.M.
Direct and sex-specific stimulation by sex steroids of creatine kinase activity and DNA synthesis in rat bone.
Proc. Natl. Acad. Sci. USA 1989, *86:* 3361-3365.

19. Sömjen D., Weisman Y., Mor Z., Harell A., Kaye A.M.
Regulation of proliferation of rat cartilage and bone by sex steroid hormones.
J. Steroid Biochem. Mol. Biol. 1991, *40:* 717-723.

20. Tremollieres F.A., Strong D.D., Baylink D.J., Mohan S.
Progesterone and promegesterone stimulate human bone cell proliferation and insulin-like growth factor-2 production.
Acta Endocrinol. 1992, *126:* 329-337.

21. Wei L.L., Leach M.W., Miner R.S., Demers L.M.
Evidence for progesterone receptors in human osteoblast-like cells.
Biochem. Biophys. Res. Commun. 1993, *195:* 525-532.

22. Malnick S.D.H., Shaer A., Soreq H., Kaye A.M.
Estrogen induced creatine kinase in the reproductive system of the immature female rat.
Endocrinology 1983, *119:* 1907-1909.

23. Kaye A.M., Weisman Y., Harell A., Sömjen D.
Hormonal stimulation of bone proliferation.
J. Steroid Biochem. Mol. Biol. 1990, *37:* 431-435.

24. Kaye A.M., Reiss N.A., Weisman Y., Binderman I.
Hormonal regulation of creatine kinase BB.
In: N. Brautbar (Ed.), Myocardial and skeletal muscle bioenergetics.
Plenum, New York, 1986, p. 83.

25. Sömjen D., Kaye A.M., Binderman I.
Stimulation of creatine kinase BB activity by parathyroid hormone and by prostaglandins in cultured bone cells.
Biochem. J. 1985, *225:* 591-596.

26. Kaye A.M., Kim T.-Y., Kohen F., Sömjen D.
Anabolic effects of estrogen and parathyroid hormone on skeletal tissues: the use of creatine kinase B activity as a response marker.
Arch. Gerontol. Geriatr. 1997, *24:* 197-209.

27. Sömjen D., Mor Z., Kaye M.
Age dependence and modulation by gonadoectomy of rat diaphyseal bone to gonadal ste-roids.
Endocrinology 1994, *134:* 809-814.

28. Berger E.
Control mechanisms in development of osteogenic and hematopoietic tissues and the use of irradiation to study them.
Unpublished Ph.D. thesis, submitted to Tel-Aviv University, 1996.

29. Fouernier B., Haring S., Kaye A. M., Sömjen D.
Stimulation of creatine kinase specific activity in human osteoblast and endometrial cells by estrogens and anti-estrogens and its modulation by calciotropic hormones.
J. Endocrinol. 1996, *150:* 275-285.

30. Weisman Y., Jaccard N., Hefferan T., Robey P., Cassarola F., Kaye M.A., Sömjen D.
Sex specific response of bone cells to sex steroids in vitro and its modification by prenatal androgenization.
J. Bone Miner. Res. 1990, *5:* S219.

31. Nasatzky E., Schwartz Z., Boyan B.D., Soskolne W.A., Ornoy A.
Sex-dependent effects of 17-beta-estradiol on chondrocyte differentiation in culture.
J. Cell Physiol. 1993, *154:* 359-367.

32. Katzburg S., Lieberherr M., Ornoy A., Klein B.Y., Hendel D., Sömjen D.
Isolation and hormonal responsiveness of primary cultures of human bone-derived cells: gender and age differences.
Bone 1999, *25:* 667-673.

33. Beresford J.N., Galaghar J.A., Russel R.G.G.
Production of osteocalcin by human bone cells in

vitro. Effects of 1,25$(OH)_2D_3$, Parathyroid hormone and glucocorticoids.
Metab. Bone Dis. Rel. Res. 1984, *5:* 229-234.

34. Bradford M.
A rapid and sensitive method for the quantification of microtram quantities of protein utilizing the principle of protein-dye binding.
Anal. Biochem. 1976, *72:* 248-254.

35. Ankrom M.A., Patterson J.A., d'Avis P.Y., Vetter U.K., Blackman M.R., Sponseller P.D., Tayback M., Gehron Robey P., Shapiro J.R., Fedarko N.S.
Age-related changes in human oestrogen receptor α function and levels in osteoblasts.
Biochem. J. 1998, *333:* 787-794.

36. Onoe Y., Miyaura C., Ohta H., Nozawa S., Suda T.
Expression of estrogen receptor beta in rat bone.
Endocrynology 1997, *138:* 4509-4512.

37. Longcope C.
Adrenal and gonadal estrogen secretion in normal females.
J. Endocrinol. Metab. 1986, *65:* 213-228.

38. Kalu D.N., Salerno E., Liu C.C., Echon R., Ray M., Garza-Zapata M., Hollis B.W.
A comparative study of the actions of tamoxifen, estrogen and progesterone in the ovariectomized rat.
Bone Miner. 1991, *15:* 109-124.