Journal of Cellular Biochemistry 63:115–122 (1996)

Differential Induction of Cell-Mediated Mineralization in Rat Marrow Stroma by Sera From Women of Low and High Risk for Vertebral Fracture

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Abstract The purpose of this study was to analyze the ability of sera to reflect the state of bone metabolism by testing the osteogenic response of mesenchymal cells in culture. Sera of 20 peri- and postmenopausal women were tested before the initiation of hormone replacement therapy. The responding cells were osteoprogenitors (OPC) of rat marrow stroma which normally respond to dexamethasone (DEX) and β-glycerophosphate (βGP) by proliferation, differentiation, and mineralization in culture. Instead of DEX, diluted sera (1:50) were applied to rat stromal cell cultures for analysis of their ability to affect cell proliferation, specific alkaline phosphatase (ALP) activity, and cell-mediated mineralization. The results were compared individually with the respective values of vertebral bone mineral density (BMD), expressed as the number of standard deviations above or below the mean BMD of reference populations (positive or negative Z-score). Serum donors were divided in two; the group with positive Z-scores was considered to have a low risk, and that with negative Z-scores was considered to have a higher risk for vertebral fractures. No significant difference was found between the two groups in the ability of their sera to induce cell proliferation or specific ALP activity. However, sera representing negative Z-scores induced sixteenfold less mineralization than those of positive Z-scores. The scatter of individual mineralization values was highly discriminatory between the two groups ($\alpha < 0.00$). These results indicate that the serum-induced, cell-mediated mineralization in culture might be suitable for initial evaluation of fracture risk and thus deserve further investigation.

Key words: serum, osteoporosis, alkaline phosphatase, DEXA, Z-scores, mineralization, marrow stroma

Postménopausal osteoporosis results from an acceleration of bone loss typically caused by increased bone resorption which is associated with a relatively insufficient osteoblastic response [Riggs and Melton, 1986]. Diminished estrogen levels have been implicated in causing osteoporosis [Christiansen et al., 1980]. Normally estradiol increases the proliferation of osteoblasts inducing secretion, by other tissues, of bone-cell stimulating cytokines [Manolagas and Jilka, 1995]. It might also directly stimulate osteoblastic precursors since estrogen receptors were found in osteoblast-like cells [Eriksen et al., 1988]. In addition, estradiol attenuates secretion, by extraosseous cells, of osteoclast-activating and bone-resorbing cytokines such as interleukin-1 [Pacifici et al., 1987] and tumour necrosis factor [Ralston et al., 1990]. Estradiol also inhibits production of interleukin-6 which is a bone-resorbing cytokine [Manolagas, 1995]. Accordingly, estradiol replacement has been shown to delay menopausal bone loss [Johansen et al., 1988; Prestwood et al., 1994].

Trabecular bones, such as the vertebrae, are the first to undergo bone loss in postmenopausal osteoporosis, apparently because of their high surface area exposed to osteoclasts. Therefore, one of the diagnostic approaches to osteoporosis was to measure bone mineral density (BMD) of vertebral bodies. BMD values are compared with the mean BMD (reference value) derived from an age- and anthropometrically matched population of women. The number of standard deviations (SDs) from the mean of the appropriate reference population (negative Z-score) can serve to determine cutoff points for definition of osteopenia and osteoporosis. The use of age-matched

Received February 6, 1996; accepted April 22, 1996.

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BMD references reflects the age-dependent decrease in bone mass, whereas the fracture risk is claimed to be independent of age [Kanis et al., 1994]; therefore, to estimate the fracture risk it was recommended to count the number of SDs from the mean BMD of young age references. In this case BMD values from 1 SD to 2.5 SDs below the reference are considered osteopenic, whereas values more than 2.5 SDs below reference are considered osteoporotic [for a review on osteoporosis see WHO Study Group, 1994]. For the present study we used the age-matched references (Z-score) since they should reflect recent changes in bone mass better than the long-term changes, derived from utilization of young age references. Some women enter menopause with a higher bone mass than others [Christiansen et al., 1987] and therefore at the first couple of years, even in the face of an accelerated bone loss, may present with a "normal" bone mass. This may require a repeated measurement after 1–5 years [Riis, 1995] to calculate the rate of bone loss. Consequently, extensive efforts have been made in recent years to develop biochemical assays for real-time assessment of bone turnover rates [Delmas, 1993]. Currently, biochemical assays are based on single bone metabolites which are indicative of either bone resorption or bone formation. A promising one is the assay of urine pyridinium cross-links, a resorption end product of bone matrix [Ueberhalt et al., 1991; Garnero et al., 1995]. Diagnosis of bone turnover may also require a serum immunoassay for osteocalcin, a marker for matrix maturation, secreated mainly by osteoblasts [Brown et al., 1984].

Here we examine a bioassay which presents a different diagnostic approach to postmenopausal osteoporosis. This approach intends to take into account any redundancy existing among osteogenic stimulatory factors, including that of intracellular signal transduction. We have analyzed the ability of sera from peri- and postmenopausal women to reflect bone turnover using rat mesenchymal stem cells as osteogenic indicators. The rationale for this approach is based on the assumption that the nonbiased rat osteoprogenitor cells (OPC) should selectively respond to total bone specific stimuli, present in the serum at real time, rather than relate to single factors. We also rely on the rat OPC to be largely unresponsive to factors which are irrelevant to osteogenesis. The main question is whether proliferation and osteogenic differentiation induced by menopausal sera correlate with the Z-score of bone density measured by dual energy X-ray absorptiometry (DEXA). It followed that only for the mineralization aspect of differentiation the response of sera applied to stromal cell cultures showed a linear correlation with the BMD Z-scores. Furthermore, these results could distinguish between positive and negative Z-scores, although women with bone-affecting conditions or women using medications that affect bone metabolism were not excluded from the study.

MATERIALS AND METHODS

Patient Information

Sera of 20 women with menopausal symptoms, assigned for hormone replacement therapy (HRT), were obtained under informed consent before starting treatment. The serum donors were chosen based on their willingness to undergo a bone density test. Each patient underwent recording of a complete medical history and a physical and gynecological examination when assigned to the clinic, followed by a complete blood count, blood chemistry, liver and kidney functions, lipid and hormone profiles, and urinalysis. The mammography and PAP smear results were negative in all the patients. The presenting symptoms were mainly hot flashes, recent onset amenorrhea or dismenorrhea, muscle and back pain, and mood changes. Follow-up of two patients started immediately after they underwent bilateral oophorectomy. There was no significant correlation between serum estradiol levels and the BMD Z-scores, although all the six normal results, showing levels >100 pM, were found in women with positive Z-scores; the other six positive and the eight negative Z-scores were associated with lower levels of estradiol, <100 pM. Two women showed elevated levels of thyroid stimulation hormone; both had positive Z-scores, and one was treated with L-thyroxin.

Preparation of Menopausal Sera

Blood was drawn into a sealed vacuum glass tube, and after 15 min incubation at room temperature it was separated by centrigugation for 10 min at 1000*g;* the serum was removed with a glass pipete and frozen at $-20°C$ until use. On day 0 of the bioassay sera were thawed and diluted in ready-to-use (with 15% fetal calf serum [FCS]) OPC stimulation medium which was filter-sterilized via a 0.045 μm filter and

distributed in microtiter plates containing rat marrow stromal cells. Aliquots of this filtrate were kept at 4°C for changing of the medium during the culture period.

Osteoprogenitor Cell Differentiation in Culture

Bone marrow cell suspensions obtained from 60–80 g female Sabra rats were seeded at 10^8 cells/25 cm^2 flask. The stromal cells were obtained, as described before [Klein et al., 1992], by removing the nonadherent hematopoietic cells during the first 10 days of culture. The remaining adherent stromal cells were removed by trypsinization and plated in microtiter plates on day (−)3, relative to the day of osteoprogenitor cell (OPC) stimulation, at 5,000 cells/well. The cells were cultured in DMEM (Dulbecco's modified Eagle's medium), supplemented with 15% FCS and antibiotics, in a humid 9% CO_2 atmospher at 37°C for 3 days. Subsequently (on day 0), the ordinary medium was replaced with OPC stimulation medium which contained in addition to the 15% FCS antibiotics and glutamine also 50 μg/ml ascorbate and 1:50 sera dilutions of osteoporotic women. β-glycerophosphate (10 mM) was added on day 11 in plates designated for day 21 mineralization assay. The media were changed every 4 days. On day 11 the cells were subjected to in situ alkalin phosphatase (ALP) activity assay.

Alkaline Phosphatase Assay

ALP activity per cell in each well was analyzed in situ under very mild alkaline (pH 7.6) [Klein et al., 1993b]. The cells were washed once, in situ, with 150 mM NaCl, 50 mM Tris (pH 7.6) buffer (TBS). The ALP substrate, p-nitrophenyl phosphate (pNPP) dissolved in the same buffer, was added to each well. After incubation at 37°C for 90 min, the hydrolyzed substrate optical density (O.D.) was measured in each well by a multichannel optical densitometer with a filter of 405 nm wavelength.

Cell Counting

The cells were fixed for 10 min with 0.5% glutharaldehyde and stained with methylene blue to determine cell quantity, using the method of Goldman and Bar-Shavit [1979] as described elsewhere (Klein et al., 1993a). The stain was eluted in situ from the cells by incubating the plates with 0.1 N HCl at 37°C for 60 min. The O.D. of acid-eluted methylene blue was measured by a multichannel spectrophotometer at 620 nm, on day 11 (1.0 O.D. unit = 50,000 cells). The ratio between the units of ALP activity and the methylene blue O.D. for each well expresses ALP activity relative to cell quantity in each well (specific ALP activity).

Quantification of Mineralization

After 2 weeks of culture in OPC stimulation medium, calcium precipitates usually become alizarin-red detectable. Day 21 is optimal for measuring calcium in our system [Klein et al., 1993a,b]. To quantitate these precipitates, plates were washed twice with TBS to remove soluble calcium and then incubated in 0.5 N HCl overnight to solubilize the nonsoluble calcium. Appropriately diluted samples were measured by atomic absorption against standard samples of known calcium concentrations. The precipitates were expressed as micrograms of calcium per well. The lowest accurate detection limit was 0.05 μg calcium/ml, below which the values became less accurate.

Statistical Methods

The cell counts and ALP activity/cell are presented as the mean ± SE, and for each serum n = 20 wells. To calculate specific ALP activity/well for each serum, the total ALP activity in each of the 20 wells of the upper quarter of the plate was divided by the methylene blue O.D. of the respective well in the lower quarter of the plate (e.g. A2/H2, B2/G2, etc).

Mineralization is presented as water nonsoluble (but acid-soluble) calcium, mean micrograms ± SE calcium/well, for each serum n = 20 wells.

Assignment of the women to the osteopenic and nonosteopenic groups was done according to the negativity or positivity of Z-scores calculated by the software of the DEXA device. Z-scores expressed the number of standard deviations of each bone mineral content value from that of the mean normal value of women matched by age and body dimensions. The Z-score values, especially the negative scores, represent the risk for developing osteoporosis and fractures. Regression lines and the correlation between various parameters were derived using Cricket Graphics software on a Macintosh computer, and their significance was obtained using the critical values table of Pearson r coefficient by a two-tailed test. The ability to distinguish between members of osteopenic and nonosteope-

TABLE I. Induction of ALP Activity and Mineralization in Rat Marrow Stroma by Sera of Perimenopausal Women Compared With BMD Z-Scores

Serum donor Number	Age	Years in menopause	DEXA Z-score	ALP[a] activity	Cells[b]/well	Calcium (μg/well)	Remarks
1	53	0	+0.1	9.551 ± 0.250	0.703 ± 0.011	4.440 ± 0.315	L-thyroxine for hypothyroidism
2	63	13	−0.2	9.032 ± 0.386	0.658 ± 0.024	1.710 ± 0.259	
3	53	6	−2.0	11.388 ± 0.275	0.636 ± 0.014	1.105 ± 0.288	
4	47	0	+1.7	8.566 ± 0.242	0.738 ± 0.020	2.685 ± 0.151	
5	66	13	−1.4	10.139 ± 0.253	0.712 ± 0.017	0.645 ± 0.055	Untreated stomach cancer
6	63	10	−0.6	11.268 ± 0.556	0.510 ± 0.025	0.660 ± 0.048	Steroids[c]
7	50	6	−1.2	9.712 ± 0.424	0.711 ± 0.033	0.340 ± 0.054	
8	50	0	+2.3	7.265 ± 0.243	0.912 ± 0.026	10.010 ± 0.614	Premenopausal
9	50	0.3	+0.5	8.245 ± 0.219	0.857 ± 0.008	9.602 ± 0.334	Thyroid nodule, early menopause
10	57	7	−0.4	30.940 ± 3.955	0.889 ± 0.010	0.010 ± 0.000	Peptic ulcer
11	50	0.5	+0.5	7.034 ± 0.389	0.913 ± 0.026	18.220 ± 1.456	
12	49	5	−1.1	41.951 ± 0.798	0.860 ± 0.006	0.001 ± 0.000	
13	55	0	+2.5	8.471 ± 0.779	1.129 ± 0.157	20.350 ± 2.484	
14	53	0.5	+0.2	8.500 ± 0.737	0.682 ± 0.010	12.890 ± 0.351	
15	54	0.5	+2.2	34.879 ± 3.000	1.082 ± 0.010	6.190 ± 0.550	
16	50	0.3	+1.4	33.333 ± 1.131	0.337 ± 0.013	12.970 ± 0.973	Bilateral oophorectomy
17	54	1	+1.3	9.104 ± 0.730	0.683 ± 0.068	3.440 ± 0.361	
18	53	0	+1.4	13.113 ± 1.075	0.403 ± 0.028	12.130 ± 0.389	
19	59	10	+1.7	8.825 ± 0.589	1.358 ± 0.035	8.568 ± 0.762	
20	48	1	−0.75	0.170 ± 0.143	0.586 ± 0.018	0.527 ± 0.121	Bilateral oophorectomy

[a]One unit = nanomoles per 90 min per 50,000 cells.
[b]One O.D unit = 50,000 cells.
[c]Treatment for chronic obstructive pulmonary disease.

nic groups was analyzed by rank scores using the nonparametric Mann-Whitney U test.

RESULTS

Table I summarizes details relevant to the peri- and postmenopausal serum donors who underwent DEXA analysis of spinal BMD and whose sera were analyzed for the ability to induce osteogenic effects in culture. Two groups are distinguished according to whether their BMD Z-scores showed negative (osteopenic, n = 8) or positive (nonosteopenic, n = 12) bone reserve. Figure 1 shows a comparison of mean bioassay parameters between the two groups. Day 11 cell counts induced by osteopenic sera were lower than those induced by nonosteopenic sera, but these differences were small and not significant. Osteopenic sera induced in rat marrow stroma higher mean specific ALP activity compared with nonosteopenic sera (Fig. 1); however, these differences also were not significant. Conversely, these osteopenic sera induced sixteenfold less (day 21) mineralization than nonosteopenic sera.

Analysis of Mineralization Response to Post- and Perimenopausal Sera

The correlation between trabecular bone density and the ability of menopausal sera to induce mineralization in culture was examined. Figure 2 shows the linear regression computed for deviation from "normal" spinal BMD and the mean calcium deposition/well under sera stimulation. It demonstrates a significant correlation coefficient ($r = 0.6519$, $P < 0.01$). The osteopenic/osteoporotic sera (negative Z-scores) invariably showed low mineralization inducibility (<2 μg calcium/well), whereas nonosteopenic sera induced higher mineralization (>2–20 μg/well) (see also Table I).

DISCUSSION

The rationale of the present work stems from the interaction between bone turnover with remote bone sites and with other tissues via the circulation. We have recently shown that sera of rats, which sustained juxtaimplant fractures, have lost ALP induction capacity in cultured rat

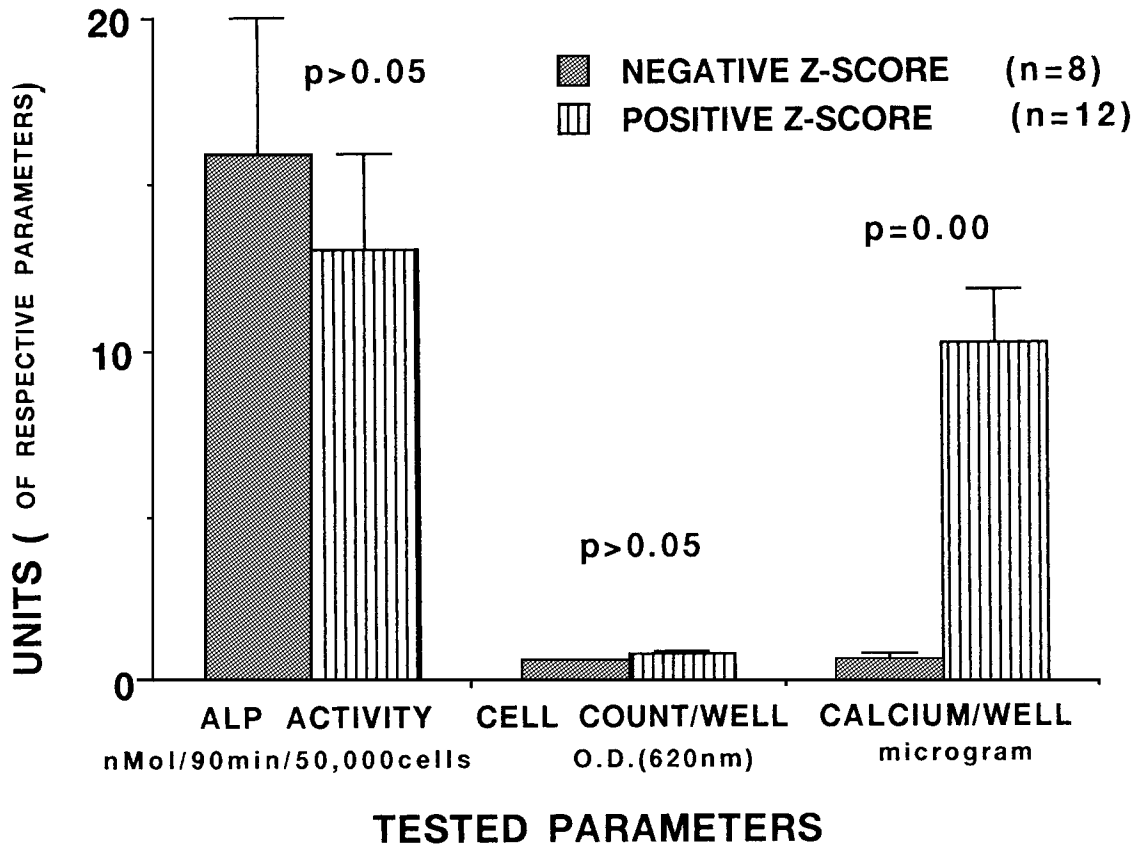

Fig. 1. Induction of ALP activity, cell proliferation, and mineralization in rat marrow stroma by women's sera. Sera, eight, with negative and twelve with positive Z-scores, diluted 1:50, were applied to the cultures on day 0. Each result is the mean ± SE of 20 microtiter wells. These results were calculated separately for sera of donors with negative and positive Z-scores; each bar represents the mean ± SE of each group. Day 11 cell count is expressed as methylene blue O.D. units (1 unit = 50,000 cells). Day 11 ALP is expressed as nanomoles per 90 min per 50,000 cells. Calcium is expressed in micrograms per well. For all three parameters, each of the 20 women (8 + 12) is represented by 20 replicas for which the private mean ± SE is given in Table I. Significance of differences between the results of negative and positive Z-scores is calculated by the Mann-Whitney U test ($\alpha > 0.05$ for cell count and ALP, and $\alpha = 0$ for mineralization).

stromal cells; this induction capacity comigrated with several macromolecular fractions of sera from rats without fractures, when applied to gel filtration columns [Klein et al., 1994]. In another study we showed that induction, by an unknown factor(s), of ALP activity in cultured stroma, using rat sera, inversely correlated with the mineralization of bone implants in the respective rats [Jaber et al., 1995]. This phenomenon may take place due to consumption of ill-defined serum molecules by the healing bone. Conversely, secretion of osteogenic factors by woven bone, formed during marrow regeneration, has been shown to increase mineralization in remote bone sites [Bab et al., 1985]. This positive systemic osteogenic effect has also been shown to comigrate with certain macromolecular fractions in gel filtration [Bab et al., 1988] in addition to a small peptide [Bab and Einhorn, 1994]. It seems that fracture healing (associated with suspected consumption of osteogenic factors) and marrow regeneration (with generation of systemic osteogenic capacity) are differently reflected in the serum. These opposing activities, of osteogenicity affected by serum are in turn different from serum activity in menopausal women at least regarding ALP induction. Whatever the trend of activity, these observations indicate that the serum could reflect bone metabolism, in a semi- or fully quantitative manner, and show a certain degree of specificity in distinguishing between these three processes (i.e., fracture healing, marrow regeneration, and osteopenia).

In the present study a rat stromal-cell model system of cell-mediated mineralization in culture was exploited to test its ability to serve as a readout for human serum-containing osteogenic

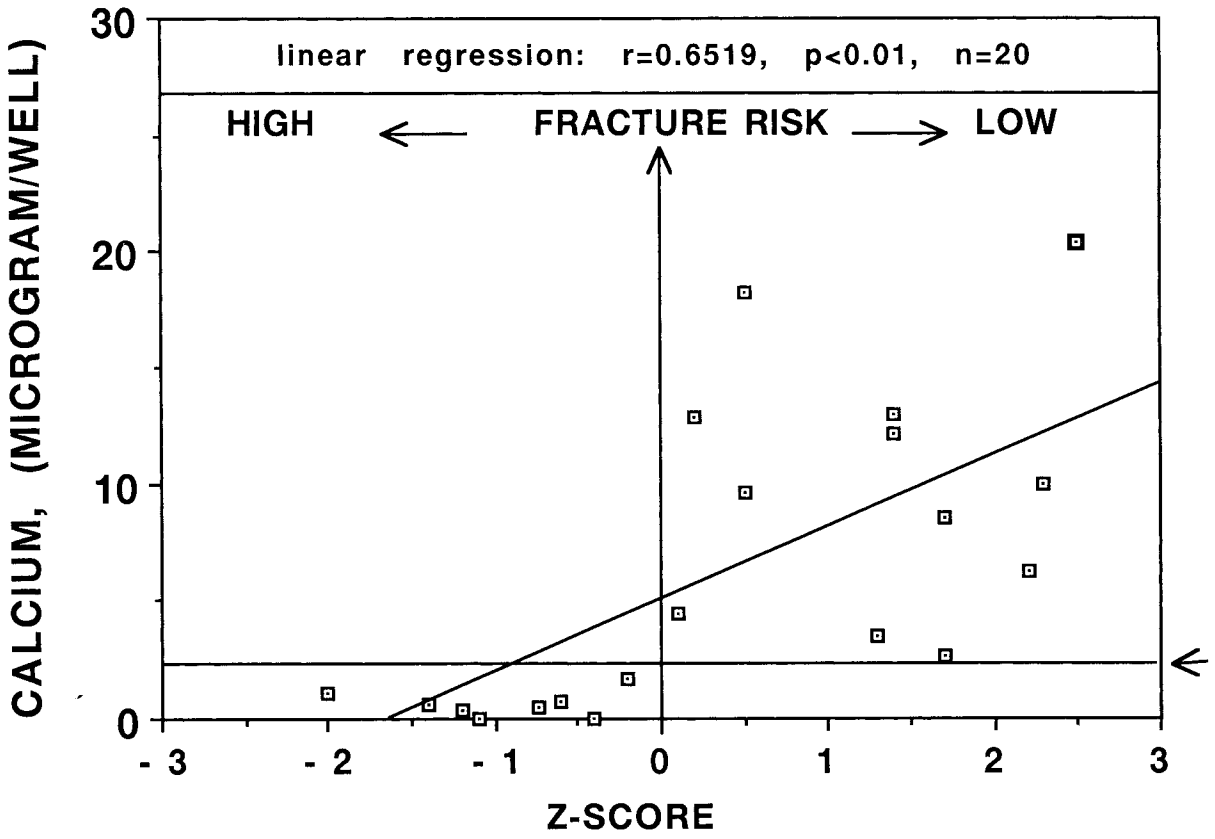

Fig. 2. Correlation between sera-induced mineralization and BMD Z-scores. Linear regression of calcium values and Z-scores both from Table I ($r = 0.6591$, $P < 0.01$). Each point represents the mean of 20 replicas for each woman's serum. The horizontal line above and parallel to the x axis (*arrow*) indicates the separation between the highest osteopenic calcium value and the lowest nonosteopenic calcium value.

stimuli. The marrow stroma is mainly a positive osteogenic indicator, although during its preparation some osteoclasts may be scraped off the endosteal surface, finding their way into the cultures, where they may have a negative effect by displacing osteoblast like cells [Ferrier et al., 1990]. No attempt was made to remove osteoclasts since no multinucleated cells were seen in these cultures. The rationale for rat cells' ability to serve as a readout for bone turnover via inducibility of bone formation is based on two reasonable assumptions. One assumes the requirement of human and rat bone cells for similar stimuli, and the other hypothesis is that osteogenic activity alone can reflect bone turnover. These assumptions seem to be consistent at least with the induction of mineralization. The most striking effect of peri- and postmenopausal sera on stromal cultures was the clear-cut difference in mineralization induction by nonosteopenic (exceedingly high) vs. osteopenic (low) calcium deposition. This characteristic could be potentially diagnostic for bone loss of postmenopausal osteoporosis, assuming that among DEXA-measured parameters the BMD Z-score is the closest measure of recent bone turnover state. Although this mineralization bioassay has clearly distinguished between the normal and bone-deficient population, the scatter of the points within each group is far from perfection. This could result from two main reasons: one is the redundency of the factors leading to osteogenic effects like mineralization and at the same time uncoupling of ALP activity and mineralization. This is reflected by the induction of ALP, which did not correlate with the DEXA-derived Z-scores, and failed to distinguish individuals with negative from those with positive Z-scores. In addition, it should be noted that the ALP assay measured pNPP hydrolysis and not bone-specific ALP protein; however, this in itself is not sufficient to explain its diagnostic inefficiency since there is extensive overlap between osteoporotic and normal results even when

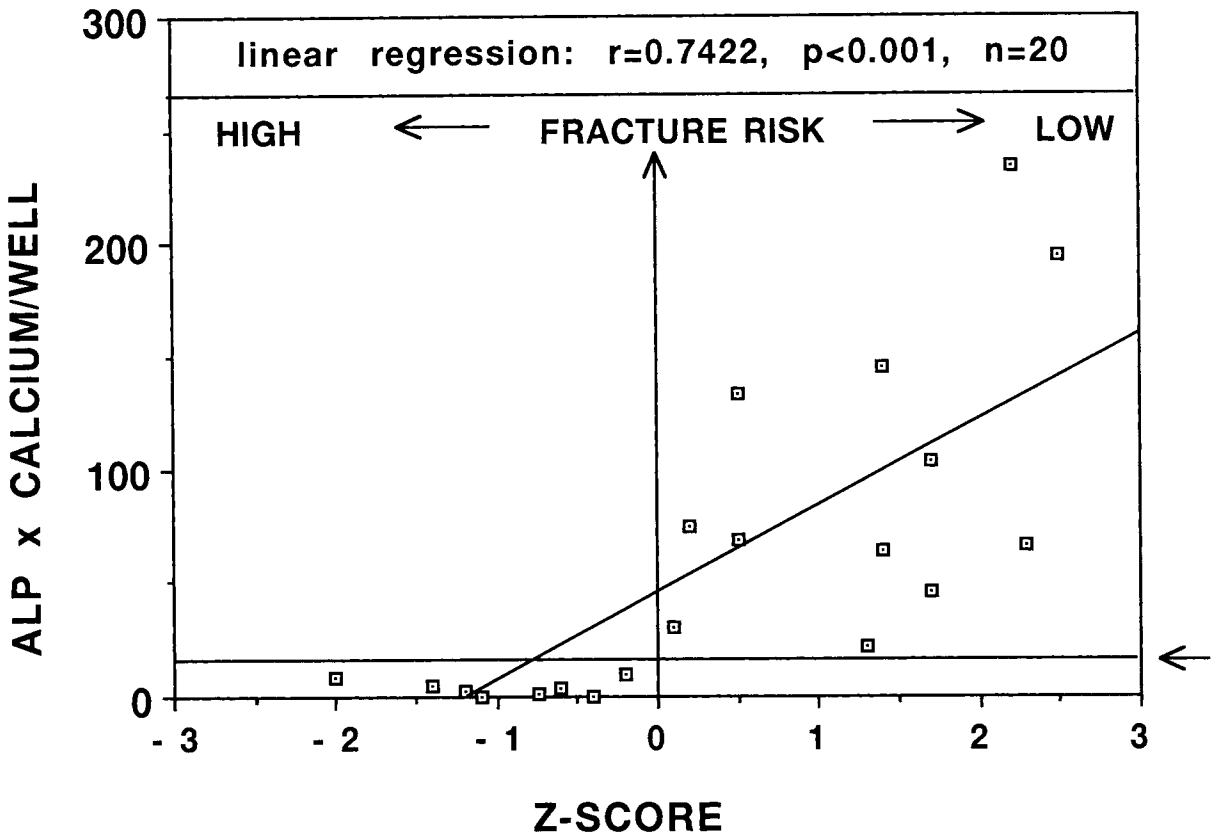

Fig. 3. Correlation between the product of sera-induced parameters (ALP/well × calcium/well) and BMD Z-scores. The correlation coefficient is improved relative to that in Fig. 2 ($r = 0.7422$, $P < 0.001$). Results of absolute ALP activity/well were obtained by multiplying the values of ALP/cell with cells/well, both from Table I. The *arrow* indicates the separation line between the values of both groups.

an anti-bone-specific ALP immunoassay is performed. Such overlapping results are encountered with other biochemical markers of osteoporosis, which supports existence of redundancy and nonspecificity among osteogenic factors. The second reason for the less than perfect correlation seen in Figure 2 could be the result of errors introduced by bone mineral density. Uneven mineralization among the three lumbar vertebrae for reasons unrelated to menopause could cause the imperfect point scatter especially among the positive Z-score group. To test the feasibility of redundancy among osteogenic factors and that the decrease in one circulating factor can be complemented by other factors, we combined the results of mineralization with ALP induction. The multiplication products of absolute ALP activity/well and calcium/well resulted in a better linear correlation with the Z-scores seen in Figure 3 than did calcium alone, as seen in Figure 2.

The data as presented do not indicate whether sera of osteopenic donors result in low mineralization levels due to lack of induction or due to inhibition. Resolution of this question requires studies with mixtures of sera from negative and positive Z-score donors. Regarding the pattern of mineralization, a pilot experiment, with one normal and one osteopenic serum, showed that the normal serum induced extracellular mineralization around and above intact cell foci. There were no dystrophic cells in these cultures, but some foci contained apoptotic cells. Conversely, cultures treated with osteopenic serum showed scattered and faint alizarin staining mostly within disintegrated cells without signs of apoptosis (not shown).

It should be noticed that serum donors were not selected by exclusion of clinical conditions that affect bone metabolism, and yet the results correlated with the Z-scores. This exhibits the applicability of mineralization induction, by the serum, to fracture risk or at least to bone turnover in menopausal women. This is an example of how culture systems for studies in differentia-

tion biochemistry can become a bioassay with clinical relevance.

ACKNOWLEDGMENTS

We acknowledge the skillful technical assistance of Mrs. Irena Gal. This work was supported by the Israel Health Ministry and the combined Hadassah/Hebrew University Medical School Common Research committee. Partial fulfillment of requirements for MsC thesis of A.M.

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