CLINICAL RESEARCH

Impaired osteoblast function in osteoporosis: comparison between calcium balance and dynamic histomorphometry

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Abstract

Osteoblast function was investigated in 27 patients with idiopathic osteoporosis. Transiliac bone biopsy specimens were taken after double labelling with tetracycline, and metabolic calcium balance was studied almost simultaneously. Many of the patients showed poor double labelling of their otherwise unremarkable trabecular osteoid, suggesting impaired formation of bone at many of these surfaces. This phenomenon was not accompanied by increased width of osteoid seams (as seen in osteomalacia), indicating that formation of the matrix and its mineralisation were in equilibrium. For the first time, highly significant positive correlations ($p < 0.01$) were found between indices of bone formation, determined by labelling with tetracycline, and calcium balance. Thus some patients with osteoporosis who are rapidly losing bone have low rates of formation of trabecular bone both by individual osteoblasts and in relation to available bone surfaces. As histological indices of bone resorption also independently correlated strongly and inversely ($p < 0.01$) with calcium balance the rate of initiation of new basic multicellular units by osteoclastic resorption of trabecular surfaces (or the depth of resorption at these surfaces) also appears to be an important determinant of mineral balance.

The mechanisms that regulate the effective life span of mature osteoblasts require further investigation, particularly as some promising treatments that can increase trabecular bone volume in osteoporosis, such as parathyroid peptide hPTH (1-34) and sodium fluoride, must work through a reversal of osteoblastic depression.

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Introduction

Osteoporosis in adults results from a defect in the remodelling of bone. Renewal of bone occurs at variable rates, being relatively rapid in iliac trabecular bone, which is therefore particularly suitable for examining the pathogenesis of loss of bone in trabecular osteoporosis. Remodelling is a sequential process in which a cohort of cells of several types cooperates.[1] Each group of cells, comprising osteoclasts, mononuclear cells, and osteoblasts, is called a basic multicellular unit (BMU).[1] Bone is first removed by osteoclasts; in iliac bone this is done to a variable depth that on average is one third the thickness of the average trabecula. After a reversal phase in which a "cement line" low in collagen is laid down, possibly by mononuclear cells,[2] formation of bone is initiated by osteoblasts recruited to the resorption surface. The cycle is completed about three months later when the osteoblasts become osteocytes.

The remodelling cycle might be disequilibrated in osteoporosis by excessive and unbalanced resorption, by an interruption in the normally well coupled recruitment process by which osteoblastic formation of bone is initiated, or by a failure of mature osteoblasts to form sufficient new bone. Such failure by the osteoblasts could be due to: an inability to synthesise sufficient bone matrix (osteoid) and then to promote its mineralisation; premature death of osteoblasts; or the early transformation of osteoblasts into their end forms the osteocytes.

By giving two short courses of demethylchlortetracycline, which binds calcium at sites of formation of new bone, fluorescence may be detected in bone several days after the end of the administration of tetracycline at sites where the antibiotic has become trapped. Our previous studies, using this technique of in vivo double labelling with tetracycline, showed a moderate, but appreciable, reduction in the rate at which new bone is formed in idiopathic osteoporosis.[3,4] We have now investigated osteoid seams that do not take up tetracycline;

reports that these are excessive in osteoporosis[5-7] have renewed interest in the controversial possibility that osteoporosis may often be the result of impaired formation of bone.[4 8-11] To investigate the importance of this unlabelled osteoid required simultaneous studies of bone metabolism, which were done by the external calcium balance technique. For the first time we could show that poor double labelling of osteoid is associated with continued loss of bone in osteoporosis.

Methods

We studied 27 patients; most of them were treated with the parathyroid peptide hPTH (1-34) after completion of this study.[12] Each freely consented to the procedures reported below, in the manner required by the hospital ethical committee. All 27 were suffering from idiopathic osteoporosis, which was diagnosed after an extensive search for secondary osteoporosis (caused, for example, by endocrinopathies, gastroenterological disease, or neoplasia) had failed to show a primary cause. Each had at least two vertebral wedge or crush fractures. Only three had been receiving drug treatment for their osteoporosis; they had been receiving replacement doses of sex hormones for at least six months before they were studied.

Studies of metabolic calcium balance were carried out as described previously.[13] Briefly, patients were admitted to a metabolic ward where they received a constant diet. After a run in of five days two or three consecutive collections of excreta were made, each taking five or six days. Each diet was chosen to simulate the patient's normal home intake of calcium and phosphate. Chromium sesquioxide was used as a continuously administered faecal marker. The precision (standard error) of the technique when done by us was about 1·4 mmol/day.[13]

Immediately before or after each balance study each patient was given a course of demethylchlortetracycline 300 mg twice daily for two days followed by an interruption of 12 days and then a further four days of treatment. Four to eight days later an 8 mm horizontal transiliac trephine biopsy specimen was obtained. The biopsy specimens were embedded without decalcification in methylmethacrylate, and serial sections of 8 μm and 20 μm thickness were cut. Five sections, each 8 μm thick, were stained with solochrome cyanine R and the proportion of trabecular surfaces covered with osteoid (OS %) and the osteoid thickness index measured.[3] Four sections were stained by Goldner's method to measure the proportion of resorption surfaces (RS %) and numbers of osteoclasts (OC) per mm^2.[3] The 20 μm thick unstained sections were examined by fluorescent microscopy, and doubly (DLS) and singly (SLS) labelled surfaces were counted and expressed in the same way as total surface osteoid with a semiautomated analyser including a digitising table (Videoplan). When a double label was seen the rate of mineralisation (M) was obtained by dividing the distance between the midpoints of the two labels (in μm) by the time interval between the midpoints of the two periods of labelling (15 days).[1]

From these data several further indices of bone formation were calculated. The rate of formation of bone per unit of trabecular surfaces (^{s}Vf) was derived according to three different formulas because there is still disagreement among bone histomorphometrists about the correct way of determining how many surfaces are actively forming trabecular bone.[14] The first formula DLS $\times$ M gave $^{s}Vf_{1}$, the second (DLS + $\frac{1}{2}$ SLS) $\times$ M gave $^{s}Vf_{2}$, and the third (DLS + SLS) $\times$ M gave $^{s}Vf_{3}$. To obtain an index of the amount of new bone made per unit surface of trabecular osteoid, values for the rate of formation of bone per unit of trabecular surface were divided by the proportion of trabecular surfaces covered with osteoid to give $^{s}Vf(BMU)_{1}$, $^{s}Vf(BMU)_{2}$, and $^{s}Vf(BMU)_{3}$, which corrects roughly for differences in numbers of basic multicellular units between patients.[15] The fraction of osteoid taking a double label (DLS/OS) was also calculated.

Nine of the biopsy specimens were technically unsatisfactory in one or more respects. In three cases, a poorly visualised tetracycline other than demethylchlortetracycline had been given. In four other cases the biopsy specimens were technically unsatisfactory for the accurate estimation of the number of osteoclasts per mm^2 and the proportion of resorption surfaces. Two further biopsy specimens were technically unsatisfactory for the measurement of osteoclasts alone. Two biopsy specimens had labels of rather indefinite extent, which were difficult to measure, but a definite absence of double labels was noted in each case.

Statistical analysis—Inspection of the data did not show any obvious differences between patients when they were grouped according to age or sex. Accordingly, the data were combined, and simple regression analysis was used to examine the relations between calcium balance and measured indices of bone formation and resorption (proportion of trabecular surfaces covered with osteoid, fraction of osteoid taking a double label, rate of formation of bone per unit of trabecular surfaces, index of the amount of new bone made per unit surface of trabecular osteoid, proportion of resorption surfaces, and number of osteoclasts per mm^2).

Multiple regression analysis was used with calcium balance relating to two independent variables. The regression equations obtained

TABLE I—*Calcium balance and histomorphometric data for 27 patients with idiopathic osteoporosis*

Case No	Sex	Age (years)	Static variables: Calcium balance (mmol/day)	Proportion of resorption surfaces (%)	No of osteoclasts/mm^2	Thickness index	Proportion of osteoid covered surfaces (%)	Dynamic variables: Rate of mineralisation (μm/day)	Proportion of doubly labelled surfaces (%)	Proportion of singly labelled surfaces (%)
1	F	68	−3·6	5·5	0·16	15·4	27·1	0·62	1·5	0·9
2	F	69	+2·9	1·0	0·09	12·6	13·9	0·73	7·8	1·7
3	M	47	−2·7	3·8	0·10	9·2	10·4	0·77	5·1	1·3
4	F	64	+0·3	2·3	0·10	13·9	18·7	0·70	10·1	4·1
5	F	75	+1·1	7·1	0·07	15·7	22·8	0·72	13·1	7·0
6	F	61	−2·9	10·7	0·24	19·0	45·4	0·85	23·7	2·0
7	F	76	+1·7	3·4	0·06	13·7	47·3	0·68	33·3	8·4
8*	M	61	−0·6	2·1	0·03	12·3	17·1	0·60	6·0	1·2
9	F	67	−0·3	9·0	0·30	15·8	21·0	0·46	12·0	2·4
10	F	53	+0·6	2·9	0·13	15·0	16·0	0·72	0·5	1·4
11†	F	70	−4·1	3·3	0·05	8·2	14·7	0·0‡	0·0	0·5
12†	F	65	−3·4	7·4	0·37	9·8	28·6	0·62	19·1	7·2
13	F	64	−5·2	5·6	0·21	12·0	28·7	0·52	0·9	21·0
14	M	53	−2·9	2·8	0·01	8·9	13·1	0·49	1·7	0·3
15†	F	50	−4·7	5·5	0·29	22·7	34·8	0·84	13·5	6·3
16*†	M	62	+1·2	4·8	0·31	13·3	49·1	0·76	37·4	1·5
17†	M	53	+2·3	1·4	0·01	11·0	31·6	0·61	2·3	3·1
18	F	65	−3·2	7·6	0·24	15·4	24·6	0·61	5·3	5·1
19	F	72	−6·5	4·6	0·13	14·7	14·7	0·0‡	0·0	NM
20†	F	77	+0·6	1·6	NM	22·3	19·7	0·48	4·6	2·6
21	F	63	−1·9	2·3	NM	14·1	17·0	0·0‡	0·0	NM
22	F	76	+2·1	NM	NM	11·5	16·1	0·50	12·7	2·6
23*†	F	58	−4·1	NM	NM	19·7	28·3	0·50	13·9	3·1
24†	F	60	+1·3	NM	NM	7·2	36·1	0·70	6·4	7·5
25	F	57	−0·1	NM	NM	15·2	23·9	NM	16·9	7·0
26	F	72	+1·4	2·4	0·02	15·8	19·2	NM	14·5	4·7
27	F	55	−2·9	3·7	0·06	11·3	6·8	NM	1·0	5·2
Mean (SD)			−1·2 (2·7)	4·4 (2·6)	0·14 (0·11)	13·9 (3·9)	24·0 (11·1)	0·64‡ (0·12)	9·8 (9·9)	4·3 (4·3)

NM = Not measurable.
*Patients treated with replacement doses of sex hormones.
†Patients previously reported on (cases 22, 26, 61, 43, 44, 6, 24, and 63, respectively).[12]
‡In absence of double labels rate of mineralisation assumed the value 0·0. These values excluded in calculating mean (SD).
Conversion: SI to traditional units—Calcium: 1 mmol ≈ 40 mg.

were tested for significance by calculating partial regression coefficients with their levels of significance.[16] Overall multiple correlation coefficients (R) were also calculated.[17]

Results

Comparison of normal data with results from our patients—The mean rate of mineralisation observed in our patients (excluding those in whom it could not be measured (table I)) was similar to that observed in our previous studies,[3] being moderately reduced compared with our normal mean (SD) finding of 0·72 (0·12) μm/day in iliac trabecular bone. If compared with the data obtained by Melsen and Mosekilde in a younger group of normal subjects,[15] values for our patients of the index of bone forming activity at the level of the individual basic multicellular unit ($^{s}Vf(BMU)_{3}$) were reduced (mean (SD) 0·32 (0·21) *v* 0·5 (0·2); Student's *t* test = 3·33; $p < 0·01$). Because, however, of the rather greater numbers of basic multicellular units in our patients compared with those of Melsen and Mosekilde there was no significant difference in bone forming activity at the tissue level ($^{s}Vf_{3}$). The proportion of trabecular surfaces covered with osteoid was, however, higher in our patients than in those of Melsen and Mosekilde (24·0 *v* 17·9%; $p < 0·05$).

Simple regression analysis—The proportion of resorption surfaces ($r = -0·40$; $p < 0·10$; $n = 23$) and numbers of osteoclasts per mm² ($r = -0·32$; $p > 0·10$; $n = 21$) correlated weakly and inversely with calcium balance. Of the variables of formation $^{s}Vf(BMU)_{1}$ correlated significantly at the 5% level with calcium balance ($r = 0·40$; $n = 24$), and $^{s}Vf(BMU)_{2}$ correlated significantly at the 10% level ($r = 0·35$; $n = 24$). The fraction of active osteoid surfaces correlated at the 2% level with calcium balance ($r = 0·47$; $n = 27$).

Multiple regression analysis—Because of the normally positive coupling mechanism between bone formation and resorption,[1] a positive relation between calcium balance and formation of bone would be obscured by a negative relation between calcium balance and resorption of bone, and vice versa. To examine these relations independently multiple regression analysis was used with calcium balance relating to two independent variables (one resorption and one formation).

The two most significant were calcium balance on the fraction of osteoid surfaces taking a double label and the proportion of resorption surfaces ($R = 0·73$; $n = 23$) and calcium balance on the fraction of osteoid surfaces taking a double label and the number of osteoclasts ($R = 0·71$; $n = 21$). The partial regression coefficients on calcium balance were significant for the fraction of osteoid surfaces taking a double label at the 0·1 and 0·5% levels respectively. The partial regression coefficients on calcium balance for proportion of resorption surfaces and number of osteoclasts per mm² were also highly significant, at the 0·5 and 1·0% levels respectively (table II).

When other indices of bone formation were substituted for the fraction of osteoid surfaces taking a double label the multiple correlation coefficients were slightly less significant, the next best variables being doubly labelled surfaces and $^{s}Vf(BMU)_{1}$. When, however, the proportion of resorption surfaces was combined with that of osteoid covered surfaces or $^{s}Vf_{1}$ the calculated values of R fell to 0·51 and 0·60 respectively.

The calculated regression equation relating calcium balance to resorption surfaces and active osteoid surfaces (table II; equation 3) suggests that to be in exact (zero) calcium balance, a patient should have measured resorption surfaces covering no more than 0·14:0·60 or 23% of active bone forming surfaces when these are measured as osteoid surfaces taking a double label. In 17 of 21 patients this ratio was exceeded, and in eight of these resorption surfaces on trabecular osteoid were more extended than double labelled bone forming surfaces (table I).

Table III shows the mean results for calcium balance in our patients after they had been divided into four groups according to whether proportions of resorption surfaces or numbers of osteoclasts per mm² were normal or high and according to whether $^{s}Vf(BMU)_{1}$ was normal or low.

Discussion

These results show that at the time of study about one third of our patients had re-established a dynamic equilibrium with normal values for histological indices of bone formation and resorption and no evidence for further loss of bone as determined by the calcium balance (table III). In the larger number of patients with low values for indices of bone forming activity, as determined by the tetracycline labelling technique, calcium balances were substantially negative. We calculated values for some indices of bone formation relative to total trabecular surfaces (rate of formation of bone per unit of trabecular surface, number of doubly labelled surfaces), which provides information on the rate at which the bone tissue is being replaced. The other indices, which are calculated relative to just those surfaces covered by osteoid (index of amount of new bone made per unit surface of trabecular osteoid, fraction of osteoid surfaces taking a double label), provide information on how actively osteoid is being mineralised and therefore relate to the metabolic activity of the individual basic multicellular units. These indices of activity of the basic multicellular units correlated slightly better with the calcium balance data. Osteoid is normally actively mineralised under the control of the osteoblasts, after which a period of less rapid mineralisation ensues during which tetracycline is no longer taken up, the osteoid layer becomes increasingly thin, and the surface osteoblasts transform to flat lining cells. These results show that many of our patients who were losing bone had an abnormally

TABLE II—*Calculated relations between calcium balance and selected indices of bone formation and resorption*

Equation No	Calcium balance (mmol/day)	Index of bone formation	Index of bone resorption	Constant	Multiple correlation coefficient R
1	Calcium balance = 5·93	DLS/OS	−0·58 RS	−1·0	R = 0·73; n = 23; p < 0·001
2	Calcium balance = 6·59	DLS/OS	−12·18 OC	−2·2	R = 0·71; n = 21; p < 0·001
3	Calcium balance = 0·14	DLS	−0·60 RS	−0·1	R = 0·66; n = 23; p < 0·01
4	Calcium balance = 7·75	$^{s}Vf(BMU)_{1}$	−0·55 RS	+0·7	R = 0·66; n = 21; p < 0·01
5	Calcium balance = 8·81	$^{s}Vf(BMU)_{1}$	−12·7 OC	+1·8	R = 0·66; n = 19; p < 0·01
6	Calcium balance = 0·16	$^{s}Vf_{1}$	−0·57 RS	+0·0	R = 0·60; n = 21; p < 0·01

Conversion: SI to traditional units—Calcium: 1 mmol ≈ 40 mg.

TABLE III—*Rates of loss of calcium (mmol/day) in patients grouped according to whether their rates of bone formation by basic multicellular units ($^{s}Vf(BMU)_{1}$) were normal or low and whether their indices of bone resorption were normal or high*

$^{s}Vf(BMU)_{1}$	Proportion of resorption surfaces (%)		Osteoclasts/mm²	
	≤6·5	>6·5	≤0·2	>0·2
≤0·25	2·1 (n = 10)	3·2 (n = 1)	1·8 (n = 9)	4·2 (n = 2)
>0·25	0·2 (n = 6)	1·4 (n = 4)	−0·7 (n = 5)	2·0 (n = 5)

Conversion: SI to traditional units—Calcium: 1 mmol ≈ 40 mg.

high ratio of unlabelled to labelled osteoid. In view of the previous results of Darby and Meunier, who found a reduced mean thickness of completed "packets" of new trabecular bone in osteoporosis,[4] and the fairly small reduction in rates of mineralisation in these patients, the probable explanation is that the time span during which osteoid will take a double label is reduced, implying a reduced effective life span of the osteoblasts. This is compatible with our previous work which showed reduced rates of kinetically estimated formation of bone in patients showing negative calcium balance.[11]

The equally strong and independent inverse association of calcium balance with the proportion of resorption surfaces and numbers of osteoclasts per mm^2 must also be explained in the light of current concepts of the mechanism of bone remodelling. In the quantal concept introduced by Frost this process occurs simultaneously at several skeletal sites.[1] At each site the process is under the control of a single basic multicellular unit. In health, at some sites the net outcome is a gain in bone and at others a loss. By the process of remodelling bone can repair microscopic damage due to fatigue processes and adapt, if necessary, to changing forces applied to the skeleton.[18]

During actively developing osteoporosis most basic multicellular units must lose bone. If each basic multicellular unit has a fixed impairment of its ability to replace the bone resorbed, the rate at which bone is lost from the skeleton as a whole will depend not only on the degree of this impairment at the level of the basic multicellular units but also on the number of basic multicellular units active in bone at that time. Furthermore, it has been suggested that resorption of bone at individual basic multicellular units may be abnormally increased in postmenopausal osteoporosis.[19] We have no way of knowing, therefore, whether the association of the indices of resorption with negative calcium balance is the result of an increased duration and therefore extent of resorption at individual basic multicellular units or whether some external factor is initiating an increased birth rate of new basic multicellular units that each resorb a normal amount of bone.[20 21]

Whichever of these possibilities is the case, the result in some patients (about a quarter of our group (table III)) is a "high turnover" state that, unless osteoporosis is to ensue, requires a more exact matching of osteoblastic bone formation to bone resorption. Clearly, many of our patients are unable to match their resorption of bone in this way, and for them the potential advantage of a vigorous renewal of bone tissue is outweighed by the more rapidly deleterious effect this has on their skeletal mass, on which the strength of their bone substantially depends. Whether these patients with a high turnover of bone represent a separate subpopulation or whether we are viewing a single disease at different stages in its evolution[20] can only be clarified by future studies obtaining biopsy specimens at different times.

Further detailed microscopical studies of the osteoblasts are needed to compare the morphology of those cells on osteoid surfaces that do and do not promote the uptake of double tetracycline labels. A certain proportion of surfaces, less than one third using our labelling protocol, may be expected to take only a single label because, in the interval between labellings, of formation of bone being either started or stopped in the natural evolution of the healthy basic multicellular unit, and this hypothesis has recently been validated in healthy animals.[22]

Similar studies of iliac histomorphometry, with serial densitometry in peripheral cortical bone, would also help determine whether the variations in turnover of axial trabecular bone are paralleled elsewhere in the skeleton in osteoporosis, as cross sectional data suggest that in some patients osteoporosis may be localised to the axial skeleton.

Whereas a considerable amount is already known of the factors that regulate the initiation of new basic multicellular units, this study emphasises that much further knowledge is required of the factors that regulate the effective life span of the osteoblast. This can clearly be depressed in osteoporosis, yet in our therapeutic trial with the parathyroid peptide hPTH (1-34) given by daily injections[12] the substantial increases in volume of trabecular bone with unchanged rates of mineralisation seen in a similar group of patients can have resulted only from an exceptional prolongation of the effective life span of the osteoblasts. Thus the regulation of the life span of osteoblasts and its potential for therapeutic manipulation by agents such as hPTH (1-34) and sodium fluoride[23] is one of the most exciting areas for future investigation in the bone wasting diseases.

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