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Comparative Effects of Clodronate and Calcitonin on Bone in Metastatic Breast Cancer: A Histomorphometric Study

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We studied the effects of long-term treatment with clodronate, calcitonin or placebo on bone in 36 normocalcaemic women with osteolytic metastases due to breast cancer. Clodronate (1.6 g daily given to 12 patients) induced a significant decrease in osteoclast surface and osteoclast number, and a significant fall in serum calcium and urinary excretion of calcium and hydroxyproline, an effect not noted after treatment with calcitonin (100 U in 12 patients) or in 12 placebo-treated patients. Treatment with clodronate did not abnormally suppress bone turnover nor impair mineralisation, as measured by bone formation and mineral apposition rates.
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INTRODUCTION

DESPITE RECENT advances in hormonal and cytotoxic treatment for breast cancer, bone pain, fractures and hypercalcaemia are important causes of morbidity in patients with metastatic disease. These complications arise because of progressive focal or generalised osteolysis, which is usually mediated by the activation of osteoclasts either by tumour products or by products secreted by host cells in response to the presence of tumour cells [1]. The critical role of the osteoclast in mediating bone resorption provides the rationale for the use of inhibitors of bone resorption in the management of osteolytic destruction and its complications.

A number of inhibitors of bone resorption have been tested in malignant disease affecting the skeleton. These include mithramycin, gallium nitrate and non-steroidal anti-inflammatory agents, but they have been found to be ineffective or their use limited by toxicity. A great deal of recent interest has been focused on the use of bisphosphonates and calcitonins. Both types of agents are specific inhibitors of osteoclast-mediated bone resorption and both effectively reduce serum calcium in hypercalcaemia due to malignancy [2–9]. The calcium lowering effect of these agents is largely due to decreased bone resorption as judged by a fall in biochemical indices of bone resorption.

There is also clinical, biochemical and radiological evidence to indicate that the bisphosphonate, clodronate, reduces bone resorption in lytic metastatic disease in normocalcaemic patients [10–13]. Long-term experience with calcitonin has been variable in terms of the inhibition of bone resorption. No changes were reported in one study [14], but a sustained decrease in bone resorption as judged by a fall in serum calcium and urinary excretion of hydroxyproline during 6-month treatment with calcitonin has been reported by Barreca *et al.*[15]. The sustained decrease in bone resorption with clodronate is associated with a reduction in bone pain and a decrease in the extension as well as formation of new metastases in breast cancer [12, 13]. Similar effects have been noted in myelomatosis [16] and prostatic cancer [17]. These findings have stimulated the long-term use of the calcitonins and bisphosphonates in malignancies of various types in the hope that treatment might decrease the skeletal complications of neoplasia.

A potential concern with the long-term use of inhibitors of bone resorption is that they may also decrease the turnover of bone tissue. The turnover of bone is the mechanism for the self repair of bone and if this is inhibited experimentally, for example with large doses of bisphosphonate, bone fragility may increase without inducing changes in bone mass [18]. In addition, some of the bisphosphonates, particularly etidronate, also impair the mineralisation of bone [19, 20]. The potential of the bisphosphonates when used for prolonged periods depends, therefore, upon the balance between beneficial and adverse skeletal effects.

Histological data concerning long-term effects of bisphosphonates or calcitonin on bone in metastatic disease have not been reported. In this paper we report the effects of long-term treatment with clodronate and calcitonin on the histology of bone.

PATIENTS AND METHODS

The patients reported in this study comprise patients reported in two previously published studies [12, 14]. These studies examined the long-term effects of clodronate or synthetic salmon calcitonin on the clinical and radiographic expression of metastatic skeletal disease due to breast cancer. Both studies were double blind placebo controlled studies. The patients were all normocalcaemic women with multiple osteolytic bone metastases as judged by skeletal radiography and radionucleide scans, but without evidence of visceral metastases. The criterion for enrollment into the clodronate study was the presence of progressive osteolytic metastases, whereas in the calcitonin study the entry criteria demanded stable rather than progressive disease activity. The patients in both studies were treated with hormonal manipulation, with a combination of hormonal and

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cytotoxic therapy or with cytotoxic agents alone. Treatments comprised hormonal manipulation (n = 6), a combination of hormonal and cytotoxic chemotherapy (n = 28) or cytotoxic chemotherapy alone (n = 2). Hormone therapy comprised tamoxifen, medroxyprogesterone acetate and nandrolone acetate. Cytotoxic combinations were CMF (cyclophosphamide, methotrexate and fluorouracil), CAF (cyclophosphamide, doxorubicin and fluorouracil) or mitomycin with ftorafur. At the time of randomisation the treatment regimen had been unchanged for 3 months or longer. Treatment was continued unchanged during the clodronate study, but it was changed after 3 months in the calcitonin study, if the disease had progressed.

The patients given clodronate took 3.2 g daily for 1 month followed by 1.6 g daily for 5 months or an identical placebo by mouth for 6 months. The patients given calcitonin self-administered 100 U synthetic salmon calcitonin or a saline placebo subcutaneously once daily except for a gap of 1 week in each month at the time of cytotoxic treatment. Placebo injections were discontinued after 3 months, when the code was broken. Calcitonin injections were continued for 6 – 12 months.

The patients had been previously randomised as follows: 17 patients were given clodronate and 17 patients given placebo, 25 patients were given calcitonin and 25 patients placebo. In the clodronate study, 12 patients on clodronate and 5 patients on placebo underwent a bone biopsy before and after 6 months treatment. 12 patients on calcitonin and 7 patients on placebo were biopsied before and 6–12 months after the onset of treatment. Because there were no significant differences between the values measured in the placebo groups on clodronate and calcitonin, in this study, the biopsies were pooled together to serve as a control (untreated) group of 12 patients rebiopsied 6–12 months (mean = 8.9) after the onset of treatment with placebo. Thus, the groups examined in the present study were: 12 patients (mean age 59 years, range 43–66) on clodronate for 6 months, 12 patients (mean age 57 years, range 44–69) on calcitonin for an average of 9.3 months and 12 patients (mean age 54 years, range 34–71) on placebo.

The studies had the prior approval of the local ethics committees.

Histological assessment

Bone biopsies were taken from the anterior ilium under local anaesthesia using a Bordier trephine (6 mm internal diameter). None of the biopsy specimens were taken from sites previously irradiated. In addition, patients in the clodronate study underwent *in vivo* tetracycline labelling using 300 mg of dimethylchlortetracycline given twice daily for 4 consecutive days on two occasions separated by a 10-day interval. Biopsy samples were fixed in 4% formalin at pH 7.4, dehydrated with alcohol and embedded in methylmethacrylate without prior decalcification. Sections were cut using the Jung K microtome. Seven micron sections were stained with Toluidine blue and Goldner's trichrome, 14 micron sections were mounted unstained for fluorescence microscopy. The following histological measurements were made on trabecular bone utilising a semiautomated technique with side-arm attachment to the microscope and a digitizing tablet from which surfaces and areas were automatically computed. The measurements comprised:

— Bone volume (BV/TV). The proportion (%) of tissue volume (TV) occupied by both mineralised and unmineralised bone.

— Osteoid surface (OS/BS). The proportion (%) of the trabecular bone surface (BS) occupied by unmineralised bone.

— Osteoblast number (N.Ob/BS). The number of active looking osteoblasts per millimeter of trabecular bone surface.

— Osteoid thickness (OTh). The width of osteoid seams expressed in microns and corrected for obliquity.

— Osteoid volume (OV). The unmineralised bone matrix expressed as a percentage of bone volume (OV/BV).

— Mineral apposition rate (MAR). The interval between tetracycline labels expressed in microns per day. Values were corrected for obliquity.

— Bone formation rate (BFR) was computed from MAR and the measurement of the extent of the bone surface undergoing mineralisation, which was computed from the extent of double labelling plus one half of the surface expressing a single label. The values are expressed as micrometer cubed per micrometer squared of bone surface per year.

— Eroded surface (ES/BS). The proportion (%) of the bone surface occupied by erosion, assumed to be due to bone resorption.

— Osteoclast number (N.Oc/BS). The number of multinucleated and mononucleated osteoclasts closely applied to erosion surfaces and expressed as / mm of the trabecular bone surface.

Reference values for histological measurements were calculated from a group of 20 women (mean age 56 years, range 31–70) with regional osteoporosis of the hand (algodystrophy), but no evidence of generalised osteoporosis.

Biochemistry

Biochemical measurements including serum calcium, serum phosphate (Technicon AutoAnalyzer) and alkaline phosphatase (spectrophotometry) were measured at the time of the first and second biopsies. Serum calcium values were adjusted to an albumin value of 46 g/l [21]. Fasting urine was collected for 2 h after a 12-h fast for measurement of calcium, creatinine and hydroxyproline [22]. Calcium and hydroxyproline excretion were expressed as a ratio to creatinine excretion.

Statistical analysis

Results are expressed as the mean ± SEM. The significance of differences between pre- and post-treatment values are calculated using Wilcoxon's test for paired data. Comparisons of pretreatment measurements between the three treatment groups were made using Kruskall–Wallis test, except in the case of mineral apposition rate and bone formation rate, for which statistical comparison between clodronate- and placebo-treated patients was made using the Mann–Whitney test.

RESULTS

Bone history

Histomorphometric measurements from the biopsies examined are summarised in Table 1. At the time of initial assessment bone volume and osteoclast number were significantly higher in patients with cancer compared with controls (the Kruskall–Wallis test; $P < 0.05$ and $P < 0.06$, respectively). The biopsies showed a high degree of focal heterogeneity in all measured variables indicated by the high standard errors of the mean values (Table 1). This was due to the fact that some

Table 1. Histological measurements (mean ± SEM) on bone biopsies before and after treatment with clodronate for 6 months, calcitonins or placebo for 6–12 months

	Controls	Clodronate (n = 12)		Calcitonin (n = 12)		Placebo (n= 12)	
		Before	After	Before	After	Before	After
BV/TV (%)	17.4 ± 0.9	26.4 ± 4.5	17.2 ± 2.7	18.2 ± 2.7	21.6 ± 2.8	23.0 ± 1.3	18.0 ± 1.5
OS/BS (%)	7.3 ± 1.5	12.1 ± 3.7	21.4 ± 7.3	15.2 ± 2.6	14.5 ± 6.3	15.5 ± 4.5	9.0 ± 2.0
N.Ob (/mmBS)	0.47 ± 0.12	1.35 ± 0.54	1.51 ± 0.72	0.99 ± 0.28	1.87 ± 0.55	2.09 ± 0.89	1.07 ± 0.31
OTh (um)	13.3 ± 0.7	13.8 ± 1.6	16.2 ± 2.0	14.7 ± 1.1	14.3 ± 1.4	15.2 ± 1.2	12.5 ± 1.0
OV/BV (%)	2.3 ± 0.3	4.3 ± 2.1	6.1 ± 2.7	4.3 ± 0.7	5.4 ± 3.3	3.9 ± 1.4	2.3 ± 0.5
MAR (μm/day)*	0.46 ± 0.03	0.49 ± 0.04	0.55 ± 0.04	—	—	0.43 ± 0.05	0.64 ± 0.08
BFR (μm³/μm²BS/year)*	24 ± 3	37 ± 6	41 ± 4	—	—	40 ± 8	38 ± 9
ES/BS (%)	6.4 ± 0.6	9.1 ± 1.6	10.9 ± 4.2	7.9 ± 2.5	10.8 ± 2.6	8.8 ± 1.1	10.7 ± 2.7
N.Oc (/mmBS)	0.04 ± 0.01	0.23 ± 0.10	0.03 ± 0.01	0.12 ± 0.05	0.19 ± 0.06	0.19 ± 0.07	0.14 ± 0.04

There were no differences between clodronate, calcitonin and placebo treated patients at the initial assessment.
* Number of paired biopsies is 10 in clodronate and 4 in placebo.
BV = bone volume; TV = tissue volume; OS = osteoid surface; BS = bone surface; N = number; Ob = osteoblasts; OTh = osteoid thickness; OV = osteoid volume; MAR = mineral apposition rate; BFR = bone formation rate; ES = eroded surface; Oc = osteoclasts.

biopsies were taken from bone with obvious tumour involvement.

Increases in the eroded surface and osteoclast number were most marked in the proximity of metastases suggesting the focal activation of osteoclasts by tumour cells. High bone and osteoid volumes were seen only in bone with metastases, where the number of osteoblasts was also increased and the osteoid was mainly woven. Some biopsies from tumour-free bone also showed an increase in osteoid surface and osteoblast number, but osteoid volume was normal (Table 2). Bone formation rates measured at both tumour-laden and free sites were normal or slightly increased. Mineral apposition rates were consistently normal.

The numbers of tumour-involved biopsies before and after treatment were 4 and 3, 4 and 2, and 4 and 2 in patients on clodronate, calcitonin and placebo, respectively. Some involved biopsies were laden with tumour, but in others both trabecular bone involving tumour and areas of the surrounding tumour-free bone were measurable. In order to avoid the comparison of unpaired data, only similar (involved or tumour-free) areas before treatment were compared with similar areas after treatment. Paired biopsies that did not contain comparable areas of trabecular bone were excluded from analysis. Thus, statistical analyses of responses to treatment were assessable in 11 patients given clodronate, 10 patients given calcitonin and 12 patients given placebo. The distribution of tumour-involved biopsies in the groups examined was 2 out of 11, 1 out of 10 and 2 out of 12 on clodronate, calcitonin and placebo, respectively.

There were no significant differences in the indices of bone resorption between the patient groups before treatment. Treatment with placebo was associated with a small but non-significant increase in bone resorption (Fig. 1). There was no change in indices of bone resorption in calcitonin-treated patients. Treatment with clodronate was associated with a significant decrease in the number of osteoclasts, but there was no significant change in the eroded surface (Fig. 1).

The amount of osteoid varied greatly in tumour-laden bone (Fig. 1), but there were no significant changes in osteoid parameters in any of the groups during treatment. The effect of clodronate on bone formation was further examined in tumour-free bone, which showed no change in osteoid surface, thickness or volume, and no significant change in the mineral apposition rate or bone formation rate (Table 2).

Biochemistry

All the patients were normocalcaemic at the time of the first biopsy, but the serum calcium values were slightly higher and the serum phosphate values significantly higher ($P < 0.005$) in the patients treated with clodronate compared with those given calcitonin or placebo (Table 2). Urinary calcium/creatinine and

Table 2. Measurements for bone formation (mean ± SEM) in tumour-free bone on paired biopsies before and after treatment with clodronate for 6 months, calcitonins or placebo for 6–12 months

	Clodronate (n = 9)		Calcitonin (n = 9)		Placebo (n = 10)	
	Before	After	Before	After	Before	After
OS/BS (%)	9.0 ± 3.7	13.2 ± 2.7	10.6 ± 2.5	9.3 ± 2.7	9.1 ± 2.7	10.1 ± 2.5
N.Ob (1mmBS)	1.67 ± 0.73	0.91 ± 0.26	1.06 ± 0.36	1.65 ± 0.53	0.79 ± 0.26	1.18 ± 0.36
OTh (μm)	11.9 ± 0.7	13.2 ± 1.2	13.6 ± 1.1	12.9 ± 0.4	13.0 ± 1.1	12.4 ± 0.8
OV/BV (%)	2.49 ± 6.59	2.91 ± 0.77	3.26 ± 0.71	2.27 ± 0.44	1.89 ± 0.57	2.20 ± 0.53
MAR (μm/day)*	0.50 ± 0.04	0.57 ± 0.06	—	—	0.41 ± 0.7	0.62 ± 0.11
BFR (μm³/μm²BS/year)	35 ± 7	35 ± 4	—	—	46 ± 11	30 ± 17

There were no differences between the treatment groups at the initial assessment (Kruskall–Wallis test), and no significant changes in any variable during treatment (Wilcoxon test for paired data). * Number of paired biopsies is 8 in clodronate and 3 in placebo. For abbreviations—see footnote to Table 1.

Table 3. Biochemical measurements (mean ± SEM) on paired bone biopsies before and after treatment with clodronate for 6 months, calcitonin for 6–12 months or placebo for 6–12 months (Wilcoxin test for paired data)

	Normal laboratory reference range	Clodronate (n = 12)		Calcitonin (n = 12)		Placebo (n = 12)		
		Before	After	Before	After	Before	After	P1 <
Serum calcium (mmol/l)	2.20 – 2.65	2.36 ± 0.03	2.28 ± 0.04*	2.29 ± 0.03	2.27 ± 0.03	2.31 ± 0.04	ns	
Serum phosphate (mmol/l)	0.80 – 1.40	1.30 ± 0.05	1.19 ± 0.08	1.06 ± 0.05	1.12 ± 0.07	1.03 ± 0.06	1.04 ± 0.07	0.005
Serum alkaline phosphatase (U/l)	60 – 275	235 ± 21	232 ± 21	176 ± 25	224 ± 38	234 ± 36	256 ± 33	ns
Urinary calcium (mol/mol creatinine)	0.15 – 0.34	0.48 ± 0.04	0.25 ± 0.02*	0.13 ± 0.03	0.21 ± 0.04	0.19 ± 0.03	0.22 ± 0.03	0.001
Urinary hydroxyproline (μmol/mmol creatinine)	20 – 42	51 ± 2	30 ± 3*	23 ± 3	23 ± 4	40 ± 11	40 ± 121	0.001

* $P < 0.01$. P1 denotes differences between clodronate, calcitonin and placebo treated patients at initial assessment (Kruskal–Wallis test). ns = not significant.

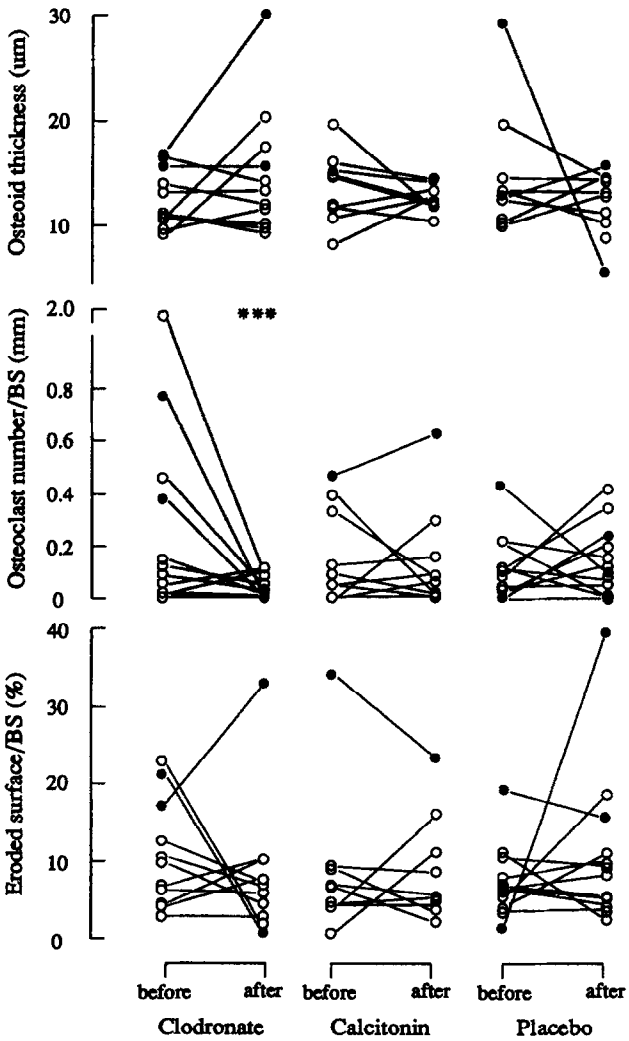

Fig. 1. Osteoid thickness, eroded surface and osteoclast number before and after treatment with clodronate for 6 months and calcitonin or placebo for 6–12 months. * * *$P < 0.03$ (Wilcoxon's test for paired data). Open and solid symbols denote tumour-free and tumour-laden bone, respectively.

hydroxyproline/creatinine ratios were also significantly higher ($P < 0.001$) in the clodronate-treated patients. Clodronate induced a significant decrease in serum calcium and urinary excretion of calcium and hydroxyproline, but there was no change in any of the biochemical measurements in patients treated with calcitonin or placebo.

DISCUSSION

The present study confirms that osteoclastic bone resorption as judged by bone histomorphometry is increased in patients with breast cancer and skeletal metastases [23–25]. Treatment with clodronate was associated with a significant decrease in bone surface occupied by osteoclasts. This supports the view that the fall in serum calcium and urinary excretion of calcium and hydroxyproline is due to a decrease in bone resorption. In contrast, calcitonin neither decreased osteoclast surface, nor altered serum calcium, urinary calcium or hydroxyproline. On the other hand, placebo-treated patients showed an increase in bone resorption, albeit not of statistical significance, which might suggest that the effects of calcitonin on bone disease were intermediate between placebo and clodronate.

These findings are consistent with beneficial short-term effects of calcitonin on bone pain [26, 27], but the less marked long-term effect of calcitonin on skeletal pain, clinical status or disease progression in these patients [14]. These effects contrast with the long-term beneficial effects of clodronate on skeletal disease [12, 13, 28]. Calcitonin decreases serum calcium levels rapidly and effectively in acute hypercalcaemia, but its longer-term effects on hypercalcaemia and bone resorption are less marked [8, 29]. The moderate effect of calcitonin on osteoclastic resorption in this study is consistent with an escape phenomenon [30], thought to be due to down regulation of calcitonin receptors of bone cells in the continued presence of calcitonin [31].

It is of interest that eroded surfaces did not decrease significantly in clodronate-treated patients. In normal bone remodelling, it is to be expected that osteoblasts would be attracted to sites of previous bone resorption by the coupling mechanism, and the inhibition of bone resorption would be expected, therefore, to decrease the eroded surface. The fact that this was not observed in our clodronate-treated patients suggests that there is uncoupling of bone formation and that sites of previous resorption may never subsequently undergo

bone formation. If true, this suggests that clodronate treatment does not restore the coupling signal between these two processes of resorption and formation.

Clodronate did not decrease mineralisation rates, which were within the normal reference range before treatment. Bone formation rates were slightly higher than normal due to the fact that many of the patients had high formation surfaces and osteosclerotic disease. Treatment with clodronate did not reduce the bone formation rate, and did not cause a significant increase in osteoid volume, indicating that clodronate does not adversely affect the mineralisation of newly formed bone.

We conclude that treatment with clodronate by mouth can induce significant and sustained decreases in osteoclastic bone resorption, and its long-term use does not adversely affect the mineralisation of newly formed bone. These properties suggest that the agent is safe and beneficial for the long-term inhibition of osteoclastic resorption in metastatic breast cancer. In contrast, salmon calcitonin given as daily injections for up to 12 months had more moderate effects on osteoclastic bone resorption.

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