Effects of heparin, histamine, and salmon calcitonin on mouse calvarial bone resorption

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SUMMARY A quantitative mouse calvarial bone resorption assay was employed to investigate the effects of the mast cell products, heparin and histamine, and of salmon calcitonin. 'Amorphous' heparin, containing a range of molecular weight fractions, inhibited resorption by 15-20% at concentrations of 0.75-5.0 mg/ml. A 'defined' heparin species of mol.wt 13 500 inhibited resorption by 14-28% at 10^{-5} - 10^{-4} mol/l. Histamine inhibited resorption by 19-55% at 10^{-3} - 10^{-2} mol/l. It is proposed that heparin and histamine depress coupled bone resorption and formation and may lead to net loss of bone. Salmon calcitonin inhibited resorption at concentrations as low as 10 pg/ml. 'Amorphous' (but not 'defined') heparin blunted calcitonin induced inhibition of bone resorption and may derepress osteoclasts.

Key words: mast cells.

Mast cells are increased throughout rheumatoid synovium, pannus, and bone erosions¹ and are the sole source of endogenous heparin² and the major source of tissue histamine.³ Chronic administration of exogenous heparin to humans, when used in the treatment of ischaemic heart disease, causes a dose related osteoporosis,⁴⁻⁸ and de Swiet *et al* have recently confirmed that heparin promotes significant bone loss in women treated for thromboembolic disease in pregnancy.⁹ The mechanism of heparin induced bone loss is uncertain. There is some evidence that heparin may stimulate the resorption of devitalised bone particles implanted subcutaneously into rats,¹⁰ but the weight of present evidence indicates that the effect of heparin is primarily antianabolic rather than catabolic. It inhibited linear growth and new bone formation in cultured neonatal mouse bone¹¹ and fracture healing in dogs and rabbits.¹² Heparin qualitatively inhibited the remodelling of cultured human trabecular bone fragments.¹³ The antiproliferative effect of heparin on bone is compatible with previous reports that it inhibits the division of a number of cell types, but smooth muscle cells most consistently.¹⁴⁻¹⁶ Preliminary studies have shown that heparin has also impaired the division of cultured isolated human trabecular bone cells.¹⁷

Histamine binding sites have been recently de-

scribed on bovine and human chondrocytes¹⁸ and on cultured isolated human trabecular bone cells,¹⁹ but little is known about the effects of histamine on articular tissues. There has been only a single report of the effect of histamine on bones in organ culture. Histamine inhibited the uptake of proline and thymidine by cultured neonatal rat bones,²⁰ which suggests that histamine, like heparin, could inhibit new bone synthesis.

Goldhaber introduced the concept that heparin may act as a cofactor of hormone action on bone.²¹ He reported that heparin potentiated mouse calvarial bone resorption mediated by parathyroid hormone (PTH), but later authors have been unable to confirm this.²² Heparin, however, stimulates intracellular cyclic adenosine-5'-monophosphate (AMP) production in cultured isolated human trabecular bone cells,²³ and this lends some weight to the hypothesis that heparin may modulate the actions of hormones on cells that are themselves often mediated by intracellular cyclic AMP. Although heparin was not found to potentiate PTH mediated increases in cyclic AMP in human bone cells, heparin inhibited calcitonin mediated rises in cyclic AMP in two cultures.²³ Endogenous calcitonin may have an important role in skeletal preservation by suppressing osteoclastic bone resorption.²⁴ If this action were inhibited locally by mast cell heparin in intact bone it could be relevant to bone loss in the rheumatoid erosion.

No quantitative assay of human bone turnover has

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yet been developed and the mouse calvarial bone resorption assay was therefore chosen to investigate these questions further.

Materials and methods

The mouse calvarial assay employed closely followed the method of Reynolds.²⁵ In each experiment 20–26 one day old outbred TO mice from no more than two litters were injected subcutaneously with 1 μ Ci of calcium-45 chloride (Amersham International, Amersham). The isotope was prepared in a standard solution of 20 μ Ci/ml phosphate buffered saline. Mice were killed when five days old by chloroform anaesthesia and decapitation. The calvarial bones (frontal with parietal bones) were isolated under a dissecting microscope and periosteal tissue preserved. Left and right hemicalvaria were separated and assigned to be paired test and control hemicalvaria. All dissection procedures were performed in a special modification of Biggar's medium (Flow, Irvine) with the addition of L-glutamine 200 mg/l and sodium bicarbonate 500 mg/l.

Paired hemicalvaria were then placed in culture in 30 mm diameter plastic Petri dishes (Sterilin, Teddington) on stainless steel platform grids. The level of medium in the dishes just reached the platform of steel mesh so that bones lay at the interface of gas and liquid phases. In each 30 mm dish 1.5 ml of medium was used containing: special modification of Biggar's medium, L-glutamine 200 mg/l, sodium bicarbonate 2.2 g/l, 5% heat inactivated fetal calf serum, penicillin 100 U/ml, streptomycin 100 μ g/ml, and amphotericin 2.5 μ g/ml. Dishes were incubated in a humidified atmosphere of CO₂ in air (1:19, v/v) at 37°C for 18 hours to allow equilibration between the bone tissue calcium concentration and that of the medium. After 18 hours medium was removed and 0.5 ml aliquots stored for later counting. Fresh control media and media with the addition of test substance(s) were added to the pairs of hemicalvaria.

Porcine heparin free from preservative was obtained from two sources: firstly, a crude commercial preparation of 168 United States Pharmacopeia units/mg (Sigma, Poole) that included heparin fractions of varying molecular weights and was possibly contaminated by up to 15% dermatan sulphate (personal communication, Dr Ian Nieduszynski, University of Lancaster). This preparation is referred to as 'amorphous heparin'. Dr Nieduszynski also kindly provided a fraction of heparin obtained after passage down a Biogel P100 column with 2.4 sulphate ester groups per disaccharide unit. This preparation of mol.wt 13 500 was free from der-

matan sulphate and is referred to as 'defined heparin'. Histamine dihydrochloride was obtained from Sigma (Poole). Salmon calcitonin (batch number P540; potency 4317 IU/mg was donated by Armour (Eastbourne). Aliquots prepared in 1 mM acetic acid were lyophilised and stored dehydrated at 2°C.

Test incubations were maintained for 48 hours, after which 0.5 ml aliquots from each dish were removed and stored for later counting. Bones were dried and then dissolved in 0.5 ml 90% formic acid for 72 hours. Scintillation fluid (Ultrafluor, USA) was added to aliquots (9:1) and each specimen counted. The following results (disintegrations per minute, dpm) were recorded: equilibrative dpm (18 hour incubation); test dpm (48 hour incubation); dissolved bone dpm; and the total dpm (equilibrative + test + dissolved bone). Then the percentage ⁴⁵Ca release (percentage bone resorption) could be obtained from the equation:

$$\text{Percentage release } ^{45}\text{Ca} = \frac{\text{test dpm}}{\text{total dpm}} \times 100$$

All test conditions and controls were performed on quintuplet or sextuplet pairs. Statistical analysis was by paired *t* tests of the differences between test and control hemicalvaria. Unpaired *t* tests were employed when comparisons were made between percentage bone resorption with calcitonin alone and with calcitonin plus heparin.

Results

EFFECTS OF HEPARIN ON MOUSE CALVARIAL BONE RESORPTION

Amorphous heparin (Table 1)

No effect on bone resorption was noted at concentrations of 0.1 μ g/ml–0.5 mg/ml. A significant inhibition of bone resorption was recorded at 0.75 mg/ml ($3.75\text{--}7.5 \times 10^{-5}$ mol/l) and 5 mg/ml ($2.5\text{--}5 \times 10^{-4}$ mol/l). This represents inhibition of 18% ($p < 0.05$) and 20% (mean of two experiments: $p < 0.01$ and < 0.02) respectively. A 15% inhibition was also noted with heparin 1 mg/ml but this did not reach significance. No difference was observed with heparin 10 mg/ml ($0.5\text{--}1 \times 10^{-3}$ mol/l).

Defined heparin (Table 2)

No effects on bone resorption were found at $10^{-7}\text{--}10^{-6}$ mol/l. At 10^{-5} mol/l (0.135 mg/ml) a 14% inhibition of bone resorption was noted ($p < 0.05$). At 10^{-4} mol/l (1.35 mg/ml) a 28% inhibition was observed ($p < 0.001$). It is apparent that defined heparin is a more potent inhibitor of bone resorption than amorphous heparin on a weight basis.

Table 1 Effects of amorphous heparin on mouse calvarial bone resorption*

<i>Heparin</i>			<i>Control</i>	<i>p Value</i>
<i>Concentration</i>	<i>% Release ⁴⁵Ca</i>	<i>% Release ⁴⁵Ca</i>		
0.1 μg/ml	31.02±6.63	35.67±6.03		NS
1.0 μg/ml	33.13±3.13	32.77±2.30		NS
10.0 μg/ml	30.40±4.93	31.76±3.34		NS
0.1 mg/ml	31.36±7.98	32.79±4.71		NS
(a) 0.5 mg/ml	22.62±2.89	24.32±8.94		NS
(b) 0.5 mg/ml	28.12±6.78	26.91±5.76		NS
0.75 mg/ml	22.98±8.40	27.97±7.79		<0.05
1.0 mg/ml	23.67±2.91	27.88±6.35		NS
(a) 5.0 mg/ml	21.68±1.45	26.08±3.00		<0.01
(b) 5.0 mg/ml	13.17±0.74	17.18±2.06		<0.02
10.0 mg/ml	15.98±1.03	16.42±3.17		NS

*The effects are expressed by the percentage release of $^{45}\text{Ca}\pm\text{SD}$. The data were derived from seven separate experiments on quintuplet or sextuplet specimens.

Table 2 Effects of defined heparin on mouse calvarial bone resorption*

<i>Heparin</i>		<i>Control</i>	<i>p Value</i>
<i>Concentration</i> <i>(mol/l)</i>	<i>% Release ⁴⁵Ca</i>	<i>% Release ⁴⁵Ca</i>	
10 ⁻⁷	30.63±6.01	29.36±7.34	NS
10 ⁻⁶	28.14±5.33	29.14±2.65	NS
10 ⁻⁵	25.26±2.17	29.52±3.35	<0.05
10 ⁻⁴	23.08±4.76	32.12±5.78	<0.001

*The effects are expressed by the percentage release of $^{45}\text{Ca}\pm\text{SD}$, based on quintuplet specimens.

EFFECTS OF HISTAMINE ON MOUSE CALVARIAL BONE RESORPTION (Table 3)

Histamine dihydrochloride 10^{-9} – 10^{-4} mol/l had no effect on mouse calvarial bone resorption. Histamine 10^{-3} mol/l inhibited bone resorption by 19% ($p<0.01$) and at 10^{-2} mol/l inhibited resorption by 55% ($p<0.001$).

EFFECTS OF SALMON CALCITONIN ON MOUSE CALVARIAL BONE RESORPTION (Table 4)

Significant inhibition of resorption was achieved by all concentrations of salmon calcitonin in the range 1 $\mu\text{g/ml}$ to 10 pg/ml.

EFFECTS OF HEPARIN ON THE CALCITONIN MEDIATED INHIBITION OF MOUSE CALVARIAL BONE RESORPTION

Amorphous heparin (Fig. 1)

Calcitonin 1 $\mu\text{g/ml}$ alone and calcitonin 1 $\mu\text{g/ml}$ +heparin 0.5 mg/ml inhibited resorption 60%

Table 3 Effects of histamine on mouse calvarial bone resorption*

Histamine		Control	p Value
Concentration (mol/l)	% Release of ^{45}Ca	% Release of ^{45}Ca	
10^{-9}	25.17±4.49	31.08±6.43	NS
10^{-7}	23.65±7.05	20.99±3.44	NS
10^{-6}	27.14±3.85	27.91±3.86	NS
(a) 10^{-5}	22.69±5.24	26.92±4.03	NS
(b) 10^{-5}	20.20±5.52	22.26±5.89	NS
10^{-4}	24.98±2.47	24.46±3.62	NS
10^{-3}	22.43±2.87	27.79±3.47	<0.01
10^{-2}	11.87±1.25	26.13±3.90	<0.001

*The effects are expressed by the percentage release of $^{45}\text{Ca}\pm\text{SD}$, based on quintuplet or sextuplet specimens. The data were derived from three separate experiments.

Table 4 Effects of salmon calcitonin on mouse calvarial bone resorption*

Calcitonin		Control	p Value
Concentration	% Release ^{45}Ca	% Release ^{45}Ca	
1.0 $\mu\text{g/ml}$	15.38 $\pm$ 2.65	29.41 $\pm$ 0.77	<0.001
0.5 $\mu\text{g/ml}$	15.47 $\pm$ 1.20	30.03 $\pm$ 2.28	<0.001
0.1 $\mu\text{g/ml}$	16.03 $\pm$ 2.00	27.99 $\pm$ 2.11	<0.001
(a) 10.0 ng/ml	15.26 $\pm$ 2.15	27.80 $\pm$ 1.43	<0.001
(b) 10.0 ng/ml	17.21 $\pm$ 1.33	35.68 $\pm$ 3.65	<0.001
1.0 ng/ml	17.68 $\pm$ 2.86	33.33 $\pm$ 3.70	<0.01
(a) 0.1 ng/ml	18.86 $\pm$ 2.19	35.64 $\pm$ 1.95	<0.001
(b) 0.1 ng/ml	13.34 $\pm$ 0.86	31.10 $\pm$ 3.33	<0.001
10.0 pg/ml	19.17 $\pm$ 2.75	31.99 $\pm$ 0.94	<0.001
1.0 pg/ml	31.36 $\pm$ 6.56	33.62 $\pm$ 7.33	NS
0.1 pg/ml	34.53 $\pm$ 1.77	34.14 $\pm$ 3.92	NS
10.0 fg/ml	32.15 $\pm$ 2.76	32.26 $\pm$ 4.63	NS

*The effects are expressed by the percentage release of $^{45}\text{Ca}\pm\text{SD}$. These data were derived from four separate experiments on quintuplet specimens.

($p<0.001$) and 34% ($p<0.01$) respectively (Fig. 1a). The difference in inhibition under these two conditions was not significant and no modulatory effect of heparin 0.5 mg/ml could therefore be shown.

Calcitonin 1 $\mu\text{g/ml}$ alone and calcitonin 1 $\mu\text{g/ml}$ +heparin 0.75 mg/ml inhibited resorption 56% ($p<0.001$) and 62% ($p<0.01$) respectively (Fig. 1b). The difference was insignificant.

Calcitonin 1 $\mu\text{g/ml}$ alone and calcitonin 1 $\mu\text{g/ml}$ +heparin 1 mg/ml inhibited resorption 59% ($p<0.01$) and 51% ($p<0.001$) respectively. This blunting of calcitonin induced inhibition in the presence of heparin 1 mg/ml was significant at $p<0.05$ (Fig. 1c).

Calcitonin 1 $\mu\text{g/ml}$ alone and calcitonin 1 $\mu\text{g/ml}$ +heparin 5 mg/ml inhibited resorption 64%

Fig. 1 The effects of salmon calcitonin 1 µg/ml alone (CT), amorphous heparin 0.5–5.0 mg/ml alone (HEP), and their combination (CT+HEP) on mouse calvarial resorption are compared with control (C) values. All data are based on quintuplet or sextuplet pairs and results are expressed as means ± standard errors of means. Significant inhibition of calcitonin induced inhibition of bone resorption was achieved by amorphous heparin 1–5 mg/ml (Figs 1c and 1d).

Table 5 Lack of effect of defined heparin on the calcitonin mediated inhibition of mouse calvarial bone resorption*

Calcitonin concentration (pg/ml)	Heparin concentration (mol/l)	Calcitonin (+heparin) % release ^{45}Ca	Control % release ^{45}Ca	p Value
100	—	13.34±0.86	31.10±3.33	<0.001
100	10 ⁻⁴	12.60±0.46	33.25±1.82	<0.001
100	10 ⁻⁵	14.36±2.65	35.96±2.98	<0.001
100	10 ⁻⁶	11.90±0.68	30.85±3.07	<0.001

*Values are expressed as the percentage release of ^{45}Ca ±SD, based on quintuplet specimens.

($p<0.001$) and 42% ($p<0.001$) respectively. This inhibition of calcitonin induced inhibition of bone resorption was significant at $p<0.001$ (Fig. 1d).

Defined heparin (Table 5)

Salmon calcitonin 100 pg/ml inhibited bone resorption 57% ($p<0.001$) (Table 4). When bones were incubated in salmon calcitonin 100 pg/ml in the presence of defined heparin 10⁻⁶–10⁻⁴ mol/l no modulation of calcitonin induced inhibition was found.

Discussion

The rheumatoid bone erosion is characterised by active osteoclastic bone resorption of juxta-articular bone. Less attention is paid, however, to the coupled process of new bone formation and attempted repair which occurs on the opposite side of a bone surface undergoing resorption.^{17 26 27} By definition, the rheumatoid bone erosion results from net bone loss, but this could follow increased resorption or impaired new bone formation. Exogenous heparin administered to humans causes a dose dependent loss of bone,⁴⁻⁹ and most available evidence suggests that heparin acts primarily by inhibition of new bone formation.¹¹⁻¹³ The present study shows that two preparations of heparin inhibit mouse calvarial bone resorption, but this cannot be simply equated with the conclusion that they depress bone loss. Although experiments showing that heparin inhibits the uptake of proline and thymidine into bone are awaited, it is likely that heparin depresses both of the closely coupled processes of bone resorption and formation and leads to net bone loss. It is of interest that amorphous heparin 10 mg/ml exerted no effect on bone resorption. We have shown that amorphous heparin is cytotoxic to cultured human endothelial cells and foreskin fibroblasts at this but not at lower concentrations.¹⁷ Such high concentrations of heparin may grossly impair the responsiveness of bone cells.

Histamine at 10⁻³–10⁻² mol/l also inhibited bone resorption and had no cytotoxic effect on cultured

human cells.¹⁷ This finding complements a previous report that histamine at concentrations higher than 30 µg/ml (2×10⁻⁴ mol/l) inhibits the uptake of proline and thymidine by cultured neonatal rat bone.²⁰ Mast cells are found almost as commonly in proximity to osteoblastic new bone formation as to osteoclastic resorption in the rheumatoid joint,^{1 17} and it is possible that mast cell degranulation in the joint may promote net bone loss by impairment of the new bone reparative response. It has been estimated that local concentrations of histamine as high as 10⁻³ mol/l may be achieved in areas of mast cell degranulation,²⁸ and as heparin exists in approximately 100 times lower molar concentration than histamine in mast cells, local heparin concentrations of 10⁻⁵ mol/l are conceivable. These phenomena demonstrated in vitro may therefore be effective in vivo.

The great potency of salmon calcitonin in the mouse calvarial resorption assay was impressive. It inhibited resorption at concentrations as low as 10 pg/ml. This must be compared with the known plasma levels of calcitonin in normal 40–70 day old mice of 93.8±8.6 pg/ml using a radioimmunoassay of human calcitonin.²⁹ If the potencies of mouse and salmon calcitonins were similar these data would provide much sought evidence that physiological levels of calcitonin are sufficient to inhibit bone resorption and protect the skeleton. Since salmon calcitonin is more potent than human calcitonin in humans,²⁴ this would be an unreasonable assumption. It has been suggested, from findings in two cultures only, that heparin inhibits calcitonin mediated increases of cyclic AMP in isolated human trabecular bone cells.²³ There are precedents for an inhibitory role of heparin on hormone action. Heparin inhibits luteinising hormone stimulated ovarian adenylate cyclase,³⁰ and dextran sulphate, the polyanion analogue of heparin, inhibits adenylate cyclase in bovine thyroid preparations.³¹ The present studies of intact mouse bone in organ culture add some weight to the hypothesis that heparin inhibits the stabilising action of calcitonin on bone. It has been shown that calcitonin and

heparin both independently inhibit bone resorption, and it might be anticipated that their combined effect on bone resorption would be summatory. In contrast, amorphous heparin 1–5 mg/ml impaired the calcitonin mediated inhibition of bone resorption. Defined heparin failed to reproduce this effect. It is possible that the amorphous preparation composed of a range of heparin glycosaminoglycans contained the fraction or fractions capable of inhibiting calcitonin, whereas the defined preparation did not. It is conceivable that the dermatan sulphate contamination of the amorphous preparation may also be relevant. Little is known of the properties of the human mast cell heparin released *in vivo*. A heparin proteoglycan of mol.wt 60 000–100 000 has been obtained from human lung mast cells³² and human skin mastocytoma cells,³³ but it is unknown whether this represents the sole form of mast cell heparin proteoglycan. Mast cell heparin release adjacent to osteoclasts in the rheumatoid joint could impair the effect of circulating calcitonin and indirectly stimulate osteoclastic resorption. Even if this indirect catabolic effect of heparin can be confirmed, however, it is unlikely to be as important as its primary antianabolic role.

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