Heparin modulates intracellular cyclic AMP in human trabecular bone cells and adherent rheumatoid synovial cells

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SUMMARY Cells were cultured from explants of human trabecular bone excised from eight patients and incubated usually for 20 minutes with bovine parathyroid hormone, salmon calcitonin, prostaglandin E₂, or heparin. The intracellular content of cyclic AMP was measured by radioimmunoassay and was significantly increased by parathyroid hormone in four, by calcitonin in two, by prostaglandin E₂ in eight, and by heparin in seven out of eight cultures. In the two cultures containing calcitonin-responsive cells heparin inhibited the cyclic AMP response induced by calcitonin. Heparin did not affect the cyclic AMP response to parathyroid hormone or prostaglandin E₂. Heparin also increased the cyclic AMP content of cultured adherent rheumatoid synovial cells. It is proposed that, in certain situations of focal pathological bone resorption, although concentrations of circulating hormones may be normal, the local release of products such as heparin may modify the effect of hormones which regulate connective tissue homeostasis. Local changes in hormone responses could contribute to the enhanced bone resorption associated with inflammatory processes such as rheumatoid arthritis.

The biological activities of bone cells are regulated by many different factors including specific ligands, some of which are produced locally, and others, for example, calcitonin and parathyroid hormone, which arrive via the circulation. It is likely that the skeletal tissue cells involved in pathological resorption of bone adjacent to the rheumatoid pannus would also be subject to functional modulation by circulating and locally produced ligands. Soluble products released by the pannus or cellular interactions occurring between the pannus and adjacent skeletal tissue could result in localised alterations in responses of bone cells to hormones. Modulation of responses to the hormones which regulate skeletal tissue homeostasis could contribute to the focal

bone destruction which characterises the rheumatoid lesion.

Serum levels of parathyroid hormone are normal in patients with rheumatoid arthritis.¹ Although serum calcitonin levels have been reported to be reduced,² there is no direct evidence that these low levels contribute to the bone destruction which occurs in patients with rheumatoid disease. We have recently reported that cells cultured from rheumatoid synovium respond to parathyroid hormone and prostaglandin E₂ (but not calcitonin) with an increase in cyclic AMP content.³ We have also shown that prostaglandins released by rheumatoid tissue may alter sensitivity to parathyroid hormone.³ It is likely that other soluble products produced by the pannus may also modify hormone responses. For example, increased numbers of mast cells have been found in the joints of patients with rheumatoid arthritis.⁴ The proximity of these cells to regions of connective tissue resorption suggests that mast cells might participate in the erosion of cartilage⁵ and of bone.⁴ Heparin glycosaminoglycan is found solely in mast cells or extruded mast cell granules,⁶ and various preparations of heparin have been shown to

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promote bone resorption *in vivo*⁷ and *in vitro*.⁸ The similarity between local erosive hyperparathyroid bone disease and rheumatoid arthritis has been described.⁹⁻¹¹ Furthermore, parathyroidectomy may cause rheumatoid erosions to regress when hyperparathyroidism coexists with rheumatoid arthritis.¹² These observations suggest that rheumatoid erosive disease is subject to hormonal modulation. The present study was therefore designed to test the hypothesis that mast-cell-derived heparin may modify the response of bone and synovial cells to circulating hormones. Our studies show that heparin increases cyclic AMP content in bone-derived and rheumatoid synovial cells. In addition heparin selectively inhibits the cyclic AMP response to calcitonin.

Materials and methods

All culture media and other solutions used for tissue culture were obtained from Grand Island Biological Company (Grand Island, New York). Fetal calf serum was purchased from Microbiological Associates (Walkersville, Maryland), and multiwell tissue culture trays (Costar) from Data Packing Corporation (Cambridge, Massachusetts). Materials used in the cyclic AMP assay were obtained from New England Nuclear (Boston, Massachusetts). Preservative-free porcine intestinal mucosal heparin, obtained from Abbott Laboratories (North Chicago, Illinois), was used in cultures 1-5 and 8. This product subsequently became unavailable, and the similar product provided by Sigma (St Louis, Missouri) was used in cultures 6 and 7. The phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX) was purchased from Aldrich (Milwaukee, Wisconsin).

Test solutions of hormones were prepared by dissolving in incubation buffer lyophilised samples of bovine parathyroid hormone (1-84) or salmon calcitonin (Armour, 5000 MRC units/mg) provided by Dr Henry T. Keutmann, Massachusetts General Hospital. Prostaglandin E₂ was a gift from Upjohn, Kalamazoo, Michigan. Cell counts were performed throughout by Coulter counter.

Human trabecular bone culture. Human trabecular bone was obtained from eight patients detailed in Table 1. Five specimens were obtained from patients with rheumatoid arthritis at sites adjacent to involved joints. Single specimens were obtained from a patient at necropsy with no apparent joint disease and from patients with Paget's disease and cleidocranial dysostosis. Cortical bone and cartilage were excised from these specimens leaving blocks of trabecular bone uncontaminated by non-osseous soft tissue. Blocks were divided into 1-2 mm long fragments which were washed in three changes of phosphate buffered saline. Fragments were placed in 6 cm or 10 cm diameter culture plates (Falcon) in Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. They were incubated at 37°C in an atmosphere of CO₂ in air (1:20, v/v). By 7 days cellular and matrix outgrowths were visible, and by 14 days cells adherent to the culture plates were found. Plates were confluent by 30-36 days. Cells from six cultures were stained for the presence of alkaline and acid phosphatase, markers of osteoblasts and osteoclasts respectively. Between 10-80% of cells contained alkaline phosphatase activity (Fig. 1) (Table 1). Cells from cultures 2-5 were stained at third passage and cells from cultures 6 and 7 were stained at second passage. Some negatively stained

Table 1 Effects of heparin on cyclic AMP content of human trabecular bone cells

Culture number	Origin of bone	% cells stained for alkaline phosphatase	Passage number	Cyclic AMP (picomoles/1 × 10 ⁵ cells)		
				Buffer	Heparin (1 mg/ml)	Fold change
1	Normal metatarsal	—	2	7.24±3.21	18.05±4.34	+2.5
2	Metacarpal	50	1	2.12±0.64	1.27±0.28	-0.6
3	Metacarpal	40	1	1.46±0.96	3.49±0.72	+2.3
4	Distal ulna	10	2	1.61±0.51	3.73±0.51	+2.3
5	Distal ulna	10	2	3.02±0.21	4.97±0.39	+1.7
6	Distal ulna	80	1	13.72±2.90	25.38±2.65	+1.9
7	Femoral head	40	1	19.31±1.73	38.02±3.04	+2.0
8	Iliac crest	—	2	1.75±0.73	5.22±1.38	+2.9

Bone specimens 2-6 were obtained from sites adjacent to joints involved by rheumatoid arthritis. Specimen 7 was from bone involved by Paget's disease. Specimen 8 was from uninvolved bone in a patient with cleidocranial dysostosis. Cells from cultures 2-5 were stained for alkaline phosphatase at third passage and cells from cultures 6 and 7 were stained at second passage. Intracellular cyclic AMP (picomoles/1 × 10⁵ cells) is expressed ± standard error of the mean of triplicate samples. The differences between paired samples are significant at *p*<0.05.

SI conversion = mg/ml × 1000 = mg/l.

Fig. 1 Cells derived from human trabecular bone explants in culture showing the flattened multipolar morphology of the cells stained positively for alkaline phosphatase activity. (Phase contrast micrograph, $\times 280$)

cells possessed a flattened, multipolar morphology similar to that of cells stained positively for alkaline phosphatase activity, and other cells had typical fibroblast-like features. It is conceivable that cultures also contained cells of endothelial origin. About 5% of cells in culture 4 contained tartrate-resistant acid phosphatase.

Adherent rheumatoid synovial cell culture. Cultures of adherent synovial cells were prepared by dispersing cells with proteolytic enzymes as described previously.¹³

Incubations of cells with hormones and heparin. Bone and synovial cells between first and third passages were treated with trypsin (0.05%, w/v)-EDTA (0.02%, w/v) and passed into multiwell trays at concentrations of $0.5\text{--}1.0 \times 10^5$ cells/well. Between 48 and 72 hours later cells were washed gently with phosphate buffered saline containing 0.9 mM calcium and 0.5 mM magnesium. They were then incubated at 37°C with test substances in a buffer solution consisting of phosphate buffered saline, 0.9 mM calcium, 0.5 mM magnesium, 0.25% (w/v) bovine serum albumin, 0.1% (w/v) glucose, and the phosphodiesterase inhibitor, IBMX 1 mM, to inhibit cyclic AMP degradation. Incubations were for 20 minutes except in the time-course experiments. The reactions were stopped by immersing the trays in liquid nitrogen.

Assay of cyclic AMP. Multiwell trays were thawed by suspension in a boiling water bath and contents brought to dryness. 1 ml of 0.05 M sodium acetate buffer, adjusted to pH 6.2, was added to each well

and the contents scraped with a plastic policeman and then transferred to glass tubes. After centrifugation at 2°C at 3000 rpm for 10 minutes 50 μ l of supernatant was removed for estimation of cyclic AMP. A radioimmunoassay employing an 18-hour incubation at 2°C of test sample, (¹²⁵I)-labelled cyclic AMP, anticyclic AMP serum, with an acetylation step to increase sensitivity, was used.¹⁴ A standard curve was obtained by linear regression analysis of the data and test values were read directly from the curve.

Statistical analysis of results was by Student's *t* tests.

Results

Effect of heparin on human trabecular bone cells. The effect of heparin, 1 mg/ml (approximately 10^{-4} M), on the content of cyclic AMP in human trabecular bone cells is shown in Table 1. Heparin increased cyclic AMP content by an average of approximately 2-fold in seven out of eight cultures. The difference between paired samples is significant at $p < 0.05$. Although it is not possible to compare adequately the cyclic AMP content of cells from different cultures, the highest unstimulated and stimulated content was found in cells cultured from a femoral head involved by Paget's disease. It is probably coincidental that the highest cyclic AMP values were found in cultures 6 and 7, in which heparin purchased from Sigma was used, as control values were also raised proportionally, representing

a consistent approximately 2-fold stimulation by both preparations of heparin. Heparin stimulated bone cell cyclic AMP content in a dose-dependent manner between 0.025 mg/ml (2×10^{-6} M) and 2 mg/ml (2×10^{-4} M) after a 20-minute incubation

Fig. 2 The dose-dependent effect of heparin on the content of cyclic AMP of human trabecular bone cells (culture 8 in passage 3) after a 20-minute incubation. Intracellular content of cyclic AMP is expressed in picomoles/ 1×10^5 cells $\pm$ standard error of the mean of triplicates.

(Fig. 2). In a time course experiment employing heparin, 1 mg/ml, a biphasic pattern of cyclic AMP content was observed. These changes occurred in cells incubated in buffer alone and in heparin (Fig. 3). A peak stimulation of approximately 2.5-fold was observed at 5–10 minutes followed by a nadir at 20 minutes and then a gradual increase in cyclic AMP levels. (SI conversion: mg/ml $\times$ 1000 = mg/l).

Effects of hormones and their interactions with heparin on trabecular bone cells. Parathyroid hor-

Fig. 3 The intracellular content of cyclic AMP in human trabecular bone cells (culture 8 in passage 4) exposed to heparin, 1 mg/ml (closed circles) and buffer alone (open circles), for time periods between 5 and 160 minutes. Cyclic AMP is expressed in picomoles/ 1×10^5 cells $\pm$ standard error of the mean of triplicates.

Table 2 Effects of parathyroid hormone and prostaglandin E_2 on cyclic AMP content of human trabecular bone cells

Culture number	Buffer	Cyclic AMP (picomoles/ 1×10^5 cells)			
		PTH (1 μ g/ml)	p value	PGE ₂ (1 μ g/ml)	p value
1	7.24 $\pm$ 3.21	36.84 $\pm$ 3.35	<0.01	329.64 $\pm$ 18.56	<0.001
2	2.12 $\pm$ 0.64	4.00 $\pm$ 1.33	NS	135.41 $\pm$ 5.75	<0.001
3	1.46 $\pm$ 0.96	1.93 $\pm$ 0.60	NS	132.10 $\pm$ 6.77	<0.001
4	1.61 $\pm$ 0.51	2.32 $\pm$ 0.71	NS	55.52 $\pm$ 1.80	<0.001
5	3.02 $\pm$ 0.21	1.91 $\pm$ 0.05	<0.02	133.34 $\pm$ 10.35	<0.001
6	13.72 $\pm$ 2.90	26.12 $\pm$ 3.39	<0.05	290.77 $\pm$ 25.09	<0.001
7	19.31 $\pm$ 1.73	46.21 $\pm$ 3.55	<0.01	202.06 $\pm$ 3.61	<0.001
8	1.75 $\pm$ 0.73	5.14 $\pm$ 1.65	<0.05	149.82 $\pm$ 4.54	<0.001

The effects of parathyroid hormone (PTH) 1 μ g/ml and prostaglandin E_2 (PGE₂), 1 μ g/ml, on the same human trabecular bone cell cultures described in Table 1. Intracellular cyclic AMP (picomoles/ 1×10^5 cells) is expressed $\pm$ standard error of the mean of triplicates. SI conversion: μ g/ml = mg/l.

none, 1 $\mu\text{g/ml}$, raised cyclic AMP content in four out of eight cultures between 2- and 5-fold and apparently decreased it in one culture (Table 2). Prostaglandin E_2 , 1 $\mu\text{g/ml}$, stimulated cyclic AMP content between 10- and 90-fold in all eight cultures (Table 2). In cultures 1 and 4 salmon calcitonin, 1 $\mu\text{g/ml}$, raised cyclic AMP content 6-fold (Fig. 4). Incubation of these cultures containing calcitonin-responsive cells with calcitonin, 1 $\mu\text{g/ml}$, and heparin, 1 mg/ml, abolished the calcitonin-mediated rise

Fig. 4 The intracellular content of cyclic AMP in two cultures of human trabecular bone cells exposed to buffer, heparin 1 mg/ml, parathyroid hormone (PTH) 1 $\mu\text{g/ml}$, parathyroid hormone 1 $\mu\text{g/ml}$ plus heparin 1 mg/ml, salmon calcitonin (CT) 1 $\mu\text{g/ml}$, and salmon calcitonin 1 $\mu\text{g/ml}$ plus heparin 1 mg/ml. Heparin inhibited the calcitonin-induced rise in cyclic AMP in both cultures but did not inhibit the parathyroid-hormone-induced rise in culture 1. Parathyroid hormone did not raise cyclic AMP above buffer values in culture 4.

in cyclic AMP leaving the 2-fold increase induced by heparin alone. (SI conversion: $\mu\text{g/ml} = \text{mg/l}$).

To determine whether the inhibition of the calcitonin-induced cyclic AMP response by heparin was specific for this hormone cells were incubated with parathyroid hormone in the presence of heparin. As shown in Fig. 4, in culture 1 parathyroid hormone, 1 $\mu\text{g/ml}$, produced a 5-fold increase of cyclic AMP levels to 37.6 ± 3.42 picomoles/ 1×10^5 cells. Coincubation with heparin, 1 mg/ml, did not alter the parathyroid-hormone-induced increase in cyclic AMP content (35.43 ± 2.01 picomoles/ 1×10^5 cells). The effect of heparin on prostaglandin E_2 -induced cyclic AMP response was tested in two additional cultures, and no effect was observed (data not shown).

Effect of heparin on synovial cells. As was observed in the bone cells, incubation of rheumatoid synovial cells with heparin produced a dose-dependent increase in cyclic AMP content (Fig. 5). Maximal increases in cyclic AMP levels were detected with heparin, 1 mg/ml. As observed in the bone-derived cells, time course experiments em-

Fig. 5 The dose-dependent effect of heparin on the content of cyclic AMP of adherent rheumatoid synovial cells after a 20-minute incubation. Cyclic AMP is expressed in picomoles/ 1×10^5 cells $\pm$ standard error of the mean of triplicates.

ploying heparin 1 mg/ml demonstrated a biphasic pattern of changes in cyclic AMP levels.

Discussion

The chronic administration of heparin in high doses to man has been associated with severe osteopenia.⁷ A similar effect of heparin on enhancing bone resorption has been observed *in vitro*. Goldhaber,⁸ employing a mouse calvarial organ culture system, demonstrated that heparin increased bone resorption by potentiating the effects of parathyroid hormone. Our studies were designed to explore the effects of heparin on human trabecular bone cells and to investigate specifically the effects of this mast cell product on the response of the target cells to hormones which are known to regulate skeletal tissue homeostasis.

To study the effects of heparin on cells of skeletal origin we have cultured cells from human bone. An early feature of trabecular bone explants was active cellular activity at the surfaces of the explant which preceded the appearance of bone-derived cells adherent to the plate. Examination with phase contrast microscopy revealed that these surface cells appeared to be synthesising an extracellular matrix, consistent with the proposal that osteoblast-like cells were cultured by this method.¹⁵ The presence of a cyclic AMP response to parathyroid hormone in four out of our eight bone cell cultures further suggests that the outgrowth cells express osteoblast phenotype.¹⁵ These cells also show strongly positive staining for alkaline phosphatase,^{16 17} which – with parathyroid hormone responsiveness – has been considered a marker of osteoblast-like cells isolated from non-human skeletal tissues.¹⁸

We observed that heparin glycosaminoglycan increased the intracellular content of cyclic AMP in a dose-dependent manner in cultured human trabecular bone cells. The interactions of calcitonin, parathyroid hormone, and prostaglandins with their cell surface receptors are accompanied by activation of adenylate cyclase and rises in intracellular cyclic AMP, and at least some of the biological effects of these hormones are probably mediated by changes in levels of this cyclic nucleotide. It is therefore possible that heparin could have effects on the skeleton by producing increases in the cellular content of cyclic AMP.

In Goldhaber's studies⁸ it appeared that heparin increased bone resorption by augmenting parathyroid-hormone-stimulated resorption. Although heparin itself increased cellular content of cyclic AMP in our cultures of osteoblast-like cells, there was no evidence that heparin potentiated the magnitude of the cyclic AMP response to para-

thyroid hormone. An additive, but statistically not significant, effect of heparin on the parathyroid-hormone-induced response was occasionally noted.

In two bone cell cultures heparin inhibited the calcitonin-induced rise in cyclic AMP without affecting the parathyroid-hormone-mediated rise in the single culture in which both hormones elevated the cyclic AMP content. If the preliminary data provided by these two bone cell cultures are confirmed, a model may be proposed in which heparin removes the inhibition of osteoclast function imposed by circulating calcitonin and permits the unopposed resorbing action of parathyroid hormone, 1,25(OH)₂ vitamin D₃, prostaglandin E₂, or osteoclast activating factor. It is possible that cellular responses to parathyroid hormone have already been 'primed' by heparin. In man it remains to be proved, however, that endogenous calcitonin has any physiological function in the regulation of bone turnover.

There are precedents for an inhibitory role of heparin on hormone action. Heparin has been shown to inhibit luteinising-hormone-stimulated ovarian adenylate cyclase.¹⁹ Dextran sulphate, the polyanion analogue of heparin, inhibits adenylate cyclase in bovine thyroid preparations.²⁰ The mechanism of inhibition is unknown. Wolff and Cook²⁰ considered the possibility that the large negative charge of these polyelectrolytes could alter cell membrane function. Structural features other than ionic charge could be important, since the equally negatively charged polyglutamic acid was only slightly inhibitory of ovarian adenylate cyclase.¹⁹ It has recently been shown that heparin can suppress the cumulus cell-oocyte complex expansion in mice which is mediated by follicle stimulating hormone but does not do so by the inhibition of cyclic AMP production.²¹

We also report that heparin increases intracellular cyclic AMP content in adherent rheumatoid synovial cells. We have demonstrated that these cells produce large amounts of prostaglandin E₂ and collagenase,^{22 23} products implicated in pathological bone resorption. The possible role of cyclic AMP in modulating release of these products is not known. Although we have demonstrated that parathyroid hormone increases cyclic AMP content in synovial cells, we have not so far detected effects of this hormone on prostaglandin or collagenase production (Goldring SR, Dayer JM, Krane SM, personal communication). The framework of mast cell granules is the metachromatic-staining heparin proteoglycan, and it has been recently reported that rat fibroblasts which ingest extruded mast cell granules increase their own production of collagenase.²⁴ It is possible that this could be mediated by cyclic AMP

in the light of reports that cyclic AMP production may influence other secretory functions of human monocytes.²⁵ We have not yet assayed the effects of heparin on collagenase production by synovial cells.

An increase in cyclic AMP content of bone and synovial cells exposed to heparin was observed within 5–10 minutes followed by a later further increase. It is likely that the early peak of production at 5–10 minutes is associated with cellular effects of cyclic AMP such as activation of protein kinase.²⁶ Cells were routinely incubated with heparin and hormones for 20 minutes, which may have resulted in underestimation of the effect of heparin on intracellular cyclic AMP production. It is not possible to assess the concentrations of heparin likely to be achieved within the microenvironment of mast cell and adjacent bone surface, but this underestimate of heparin effect suggests that heparin may exert an effect at lower concentrations than the 2×10^{-6} – 2×10^{-4} M employed in these experiments. Although the phosphodiesterase inhibitor, IBMX, was present in these experiments, it is known that even in high concentrations this compound does not completely inhibit intracellular phosphodiesterase activity. Therefore it is possible that the heparin-induced changes in cyclic AMP levels could be related to effects on phosphodiesterase activity rather than the adenylate cyclase enzyme complex.²⁷

We have recently reported the presence of increased numbers of mast cells in the rheumatoid pannus and in bone erosions characterised by osteoclastic bone resorption.⁴ These observations suggest a possible role for heparin in pathological bone resorption. The demonstration that heparin increases basal cyclic AMP levels and modulates hormone responsiveness in trabecular bone cells and adherent rheumatoid synovial cells provides a potential mechanism of action for this mast cell product in the bone destruction associated with the rheumatoid pannus.

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