Vol. 181, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 27, 1991 Pages 67-73

OSTEOCLAST RESORPTION - STIMULATING ACTIVITY IS ASSOCIATED WITH THE OSTEOBLAST CELL SURFACE AND/OR THE EXTRACELLULAR MATRIX

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Received October 11, 1991

SUMMARY: Osteoblasts mediate much of the hormonal responsiveness of osteoclasts. We and others have found that one mechanism through which this regulation is effected is by release of osteoclast resorption-stimulating activity (ORSA) into culture supernatants. However, although hormonal responsiveness is regularly observed in co-cultures, ORSA is not always detectable in conditioned media. We show here that one explanation for this finding is that ORSA may be retained by heparin-like glycosaminoglycans (GAGs) of the cell surface or extracellular matrix of osteoblasts. We found that protease-sensitive ORSA could be extracted from monolayers of the osteoblastic cell line UMR 106 with 2M NaCl or collagenase. Production of this activity was increased in response to $1,25(OH)_2D_3$. The presence of the GAG heparin was required to reveal ORSA. Immobilisation of ORSA by GAGs may assist osteoblastic cells in the regulation of the complex patterns of osteoclastic activity observed during skeletal morphogenesis and restructuring.

Osteoblastic cells incubated with bone-resorbing hormones may release, into culture supernatants, osteoclast resorption-stimulating activity (ORSA) which directly stimulates resorption by osteoclasts disaggregated from bone (1-4). However, despite the fact that stimulation of bone resorption is reproducibly seen in co-cultures of osteoblasts and osteoclasts, stimulating activity is not always detectable in culture supernatants (5-7) and may be absent even where these are taken from hormone responsive co-cultures of osteoblastic cells with osteoclasts (K Fuller, unpublished observations). Furthermore, stimulatory supernatants have to be used at concentrations of 50 per cent or above, with further dilution leading to loss of activity (K Fuller, unpublished observations). The low levels of ORSA release suggested by these observations have proved a major impediment to its identification.

Since a number of growth factors have recently been shown to bind to glycosaminoglycans (GAGs) found on the cell surface and in the extracellular matrix (ECM) (8, 9), we decided to investigate the potential of the osteoblast-like cell line UMR 106 to produce cell- and matrix-associated ORSA and assessed the effect of the GAG heparin on this activity.

MATERIALS AND METHODS

Sodium heparin grade I, approximately 181 USP units/mg, from porcine intestinal mucosa (used at 100μg/ml), trypsin and collagenase type II (Clostridium histolyticum) were purchased from Sigma Chemical Co. (Poole, Dorset, UK). 1,25 dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) (Roche, Welwyn Garden City, Herts, UK) was dissolved in alcohol (10μM) and the stock stored at -20°C. Cloned

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hormone-responsive rat osteosarcoma cells (UMR 106 parent strain, passage 25-37) were obtained from Dr T J Martin. HEPES-buffered medium 199 (199) (Flow Laboratories, Irvine, Ayrshire, UK) was used for osteoclast isolation and sedimentation, and Eagle's Minimum Essential Medium (MEM) (Flow) used for subsequent incubations. Media were supplemented with 100IU/ml benzylpenicillin (Flow) and 100µg/ml streptomycin (Flow). All incubations were performed at 37°C in 5% CO_2 in humidified air. Slices of devitalised bovine cortical bone, employed as substrates for osteoclastic resorption, were prepared as previously described (10).

Isolation and culture of osteoclasts
Osteoclasts were disaggregated from neonatal rats as previously described (11). The cell suspensions were added to 3 wells of a 100 x 18mm multiwell petri dish (Sterilin, Teddington, Middx, UK) containing bone slices. After 10mins at 37°C the bone slices were removed, washed in 199 and placed into individual wells of a 96 well plate (Flow). They were incubated for 24hrs in a total volume of 200µl. Functional absence of osteoblastic cells in osteoclastic populations was determined by the addition of $1,25(OH)_2D_3$ (10^{-8}M) to parallel cultures (2). Experiments were designed in such a way that all variables received osteoclasts from the same suspension. For osteoclast-osteoblast co-cultures $5x10^4$ UMR 106 cells were added to osteoclasts on bone slices in microtitre wells.

Assessment of osteoclast numbers and bone resorption
After incubation, bone slices were removed from wells, fixed in formalin and stained with toluidine blue (0.1% in phosphate buffered saline (PBS)). The number of multinucleate cells on each bone slice was counted by transmitted light microscopy.

Bone resorption was assessed by scanning electron microscopy. Bone slices were immersed in 10% NaOCl for 10 min to remove cells, washed in distilled water, dried and sputter coated with gold. The entire surface of each bone slice was then examined blind in a Cambridge S90 (Cambridge Instruments, Cambridge, UK) scanning electron microscope. The number of osteoclastic excavations and the plan area of bone resorbed were recorded.

Differences between groups were analysed by means of the Student's t-test.

Preparation of NaCl and collagenase extracts of UMR 106 cell cultures
UMR 106 cells were incubated in $25cm^2$ tissue culture flasks (Flow) with MEM supplemented with fetal calf serum and non-essential amino acids (Flow). When confluent, half the volume of medium was removed and replaced with fresh MEM containing $1,25(OH)_2D_3$ (10^{-8}M final concentration) or vehicle, and cells incubated for 24 hours. Extracts were then prepared as eluates by addition of 2M NaCl (2ml/flask) for 30 min at room temperature, or as digests by incubation with collagenase (50µg/ml PBS) for 2 hrs at 37°C (12). Insoluble material was removed by centrifugation (5000g) for 20 min. NaCl extracts were dialysed (cellulose tubing, molecular weight cut off 12,000 (BDH, Dagenham, Essex, UK)) against three changes of PBS (10ml against 100ml) at 4°C over a 5 hour period before addition at 10% or v/v to osteoclasts. Collagenase extracts were assayed at 10% or 3% v/v either immediately or after they had been treated with trypsin (200µg/ml; 37°C; 15 min), heated (65°C; 30 min), freeze thawed, or kept at 4°C for 2 days. Control extracts consisted of digests/eluates prepared in an identical fashion but from UMR-free flasks (cell extract-free medium).

Separation of heparin-binding factors from extracts of UMR cell layers
Extracts of UMR cell layers, prepared as described above, were subjected to affinity chromatography on heparin-Sepharose (Pharmacia, Milton Keynes, Bucks, UK) columns. 10ml of extract was added to a column of 10ml bed volume. Non-heparin binding components were washed through with 10ml PBS. Heparin-bound factors were then eluted with 10ml of 2M NaCl, washed through with 10ml PBS, dialysed (cellulose tubing, molecular weight cut off 12,000 (BDH)) against 3 changes of PBS (10ml against 100ml) at 4°C over a 5 hour period and added at 10% v/v to osteoclasts.

Lysates of UMR 106 cells
Confluent layers of UMR 106 cells incubated for 24 hours in the presence or absence of $1,25(OH)_2D_3$ were harvested by trypsinisation, washed with PBS, resuspended in 2M NaCl/0.01M Tris-HCl, pH7.5 (2ml/$25cm^2$flask), and disrupted by three cycles of freezing and thawing followed by sonication. Lysates were centrifuged at 25,000g for 30 min then dialysed as

above against three changes of PBS before addition at 10% to osteoclasts. Comparable cell extract-free controls were prepared at the same time as lysates.

Preparation of UMR 106 conditioned medium
Confluent cultures were incubated for 24 hours with serum-free MEM in the presence or absence of $1,25(OH)_2D_3$ (10^{-8}M). Supernatants were harvested, centrifuged at 5000g and added to osteoclasts at 50% v/v with fresh medium.

RESULTS

In the absence of heparin NaCl extracts of UMR 106 cell cultures had no effect on the plan area of bone resorbed by osteoclasts disaggregated from neonatal rat bones. However, in the presence of heparin, extracts of $1,25(OH)_2D_3$-treated UMR cells caused a 2.5-fold increase in bone resorption (Fig1). This stimulation of resorption was attributable to an increase in the number of osteoclastic excavations and not their mean plan area (Table 1), a pattern similar to that seen in response to other agents (3, 13). Furthermore, the increase appeared to be due to an effect on osteoclast activity rather than being the result of improved viability: osteoclast numbers were not significantly different in test and control groups at the end of the experiments (Table 1). Extracts from vehicle-treated UMR cells caused a 1.5-fold non-significant increase in resorption in the presence of heparin. There was no enhancement of resorption in parallel cultures of isolated osteoclasts incubated with $1,25(OH)_2D_3$.

Collagenase digests of both vehicle- and $1,25(OH)_2D_3$-treated UMR cell layers also caused significantly enhanced osteoclastic bone resorption, but again only in the presence of heparin. The extent of stimulation was greater after hormone treatment (plan area of bone resorbed per bone slice ($\mu m^2 x 10^{-3}$ ± SEM) in the presence of heparin: cell extract-free medium 3.4 ± 1; UMR-$1,25(OH)_2D_3$ extract 7.9 ± 1.7*; UMR+$1,25(OH)_2D_3$ 9.9 ± 1.8**; 18 bone slices per treatment;

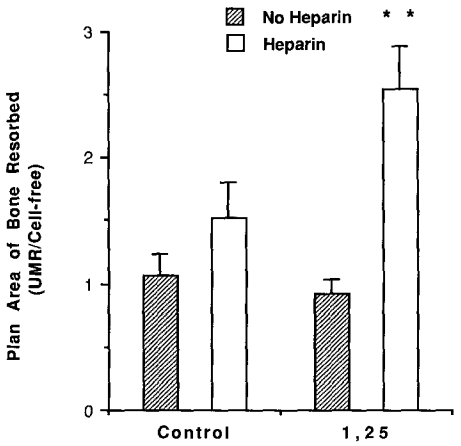

Figure 1. Bone resorption by osteoclasts incubated in NaCl extracts of UMR cells preincubated in vehicle (Control) or $1,25(OH)_2D_3$ (1,25). Results are expressed as a proportion (± SEM) of bone resorbed by osteoclasts incubated in cell extract-free medium (18 bone slices per treatment). Plan area of bone resorbed per bone slice in cell extract-free medium: 10.5 ± 2.2$\mu m^2 x 10^{-3}$. ** $p<0.01$ vs cell extract-free medium.

TABLE 1

Treatment	No. of osteoclasts per bone slice (mean ± SEM)	No. of excavations per bone slice (mean ± SEM)	Mean excavation area ($\mu m^2 \times 10^{-2} \pm$ SEM)
Cell extract-free controls	10.8 ± 0.6	3.9 ± 0.7	9.2 ± 1
UMR + $1,25(OH)_2D_3$ extracts	9.1 ± 1.3	8.2 ± 1.1 **	10.5 ± 0.7

Bone resorption by osteoclasts incubated in heparin with either NaCl extracts of UMR cells preincubated in $1,25(OH)_2D_3$ or with cell extract-free controls.
** $p < 0.01$ vs cell extract-free controls. Each figure derived from 18 bone slices.

*p<0.05, **p<0.01 vs cell extract-free medium. We found that no stimulation was detectable at 3% v/v concentration of enzyme digest (data not shown).

Heparin-potentiated activity in collagenase digests was unaffected by freeze-thawing, but was inactivated by heating to 65°C for 30 min or storage at 4°C for 2 days (Fig.2). Addition of heparin to collagenase digests before the stability studies were undertaken did not detectably enhance ORSA or protect it from inactivation (Fig.2). ORSA was lost if extracts were incubated with trypsin (200µg/ml) for 15 min at 37°C (plan area of bone resorbed per bone slice ($\mu m^2 \times 10^{-3} \pm$ SEM) in the presence of heparin: cell extract-free medium, 4.9 ± 1; UMR + $1,25(OH)_2D_3$ extract, 4.2 ± 0.8; 36 bone slices per treatment).

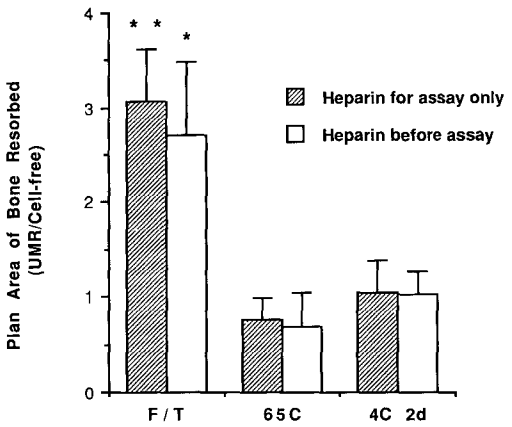

Figure 2. Bone resorption by osteoclasts incubated in heparin on bone slices, with collagenase digests of UMR cells preincubated in $1,25(OH)_2D_3$. Results are expressed as a proportion (± SEM) of bone resorbed by osteoclasts incubated with heparin in cell extract-free medium. Digests were freeze-thawed (F/T), heated to 65°C for 30 min (65C) or kept at 4°C for 2 days (4C 2d) prior to assessment of their bone-resorption stimulating activity. Heparin was added to the collagenase digests immediately after their preparation, or for the bone resorption assay only. At least 12 bone slices per variable. * p<0.05, ** p<0.01 vs appropriate cell extract-free medium control.

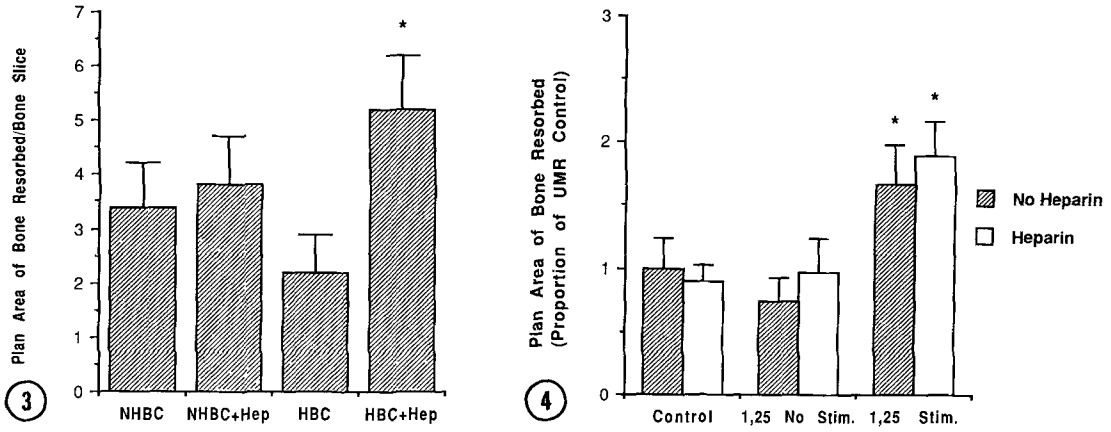

Figure 3. Bone resorption (μm^2 x 10^{-3} ± SEM) by osteoclasts incubated in the presence or absence of heparin (Hep) with either non-heparin-binding components (NHBC), or heparin-binding components (HBC) of UMR cell extracts. 12 bone slices per variable. * $p<0.01$ vs relevant heparin-free control.

Figure 4. The effect of supernatants from UMR cells which had been incubated with $1,25(OH)_2D_3$ (1,25) or vehicle (Control), on bone resorption by osteoclasts incubated in the presence or absence of heparin. Results expressed as a proportion (± SEM) of bone resorbed in the presence of supernatants from vehicle-treated UMR 106 cells. Supernatants from $1,25(OH)_2D_3$-incubated UMR cells were classified as non-stimulatory (1,25 No Stim.) or stimulatory (1,25 Stim.) based on their ability to increase resorption to a level 1.5-fold greater than that seen with supernatants from vehicle-treated UMR cells. There were at least 12 bone slices per treatment. * $p<0.05$ vs appropriate UMR control supernatant treatment.

When extracts of UMR cells were subjected to affinity chromatography on heparin-Sepharose columns, the eluate of non-heparin binding components did not stimulate resorption in the presence of heparin (Fig.3). However, heparin-binding factors, eluted from columns with 2M NaCl, had activity which caused stimulation of resorption when incubated simultaneously with heparin (Fig.3). The extent of stimulation was similar to that seen previously with unfractionated extracts. Lysates of trypsinised UMR cells had no significant effect on osteoclastic resorption, either in the presence or the absence of heparin (data not shown).

As ORSA extracted from UMR cells was heparin-dependent, the effect of heparin on co-cultures of UMR cells with osteoclasts and on ORSA in UMR-conditioned media was also assessed. The addition of heparin to UMR 106-osteoclast co-cultures resulted in a 2.4-fold ($p<0.05$) increase in osteoclastic resorption, whereas heparin was without effect in cultures of osteoclasts alone (OC + heparin/OC: 1.1 ± 0.2; 12 bone slices per treatment). Supernatants from vehicle-treated UMR cells showed no significant enhancement of resorption when added to osteoclasts. The addition of heparin to these supernatants, and to non-stimulatory and stimulatory supernatants from UMR cells incubated with $1,25(OH)_2D_3$ had no effect on subsequent resorption (Fig.4).

DISCUSSION

It is becoming increasingly clear that many growth factors become immobilised in the cell surface and extracellular matrix through binding with GAGs (8, 9, 12, 14, 15). In general, such

molecules can be eluted by salts or freed by enzymatic digestion of matrix (8, 12, 14), and show an affinity for (16-19) and have their activity increased by (19-21) the GAG heparin. We have found that 2M NaCl eluates and enzyme digests of the cloned osteosarcoma cell line UMR 106, stimulate resorption by osteoclasts when incubated in the presence of heparin. We also found that the ORSA binds to heparin-Sepharose columns, and can be eluted from them with 2M NaCl. Production of ORSA was increased by $1,25(OH)_2D_3$, a hormone which stimulates bone resorption.

These results suggest that osteoblastic cells produce an ORSA which binds to the osteoblastic cell surface or adjacent ECM. They may also account for the inconsistent detectability of ORSA in osteoblast supernatants. Furthermore, they provide the basis for a model in which extracellular binding of ORSA by GAGs may generate a reservoir of osteoclast-regulating activity. The absence of detectable activity in trypsinised UMR cell lysates suggests that ORSA may be made available when required, not through secretion from intracellular stores, but through deposition to sites in the cell surface and/or ECM from which it can be mobilised. This mobilisation may occur through neutral protease release by osteoblastic cells in response to bone-resorbing hormones (22-24); these enzymes have previously been shown to play a role in growth factor release from ECM (12, 14, 25). Mobilisation of ORSA may continue after the initiation of resorption through the action of hydrolases released by osteoclasts themselves during bone resorption (26-28).

The ORSA that we have described here may be a final common messenger for osteoclastic stimulation by osteoblasts. If so, its affinity for GAGs may facilitate localisation of stimulatory activity and enable the establishment of the complex and dynamic patterns of osteoclastic resorption observed during morphogenesis and restructuring.

ACKNOWLEDGMENT

This work was supported by National Institutes of Health grant AR39623.

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