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The regulation and enzymatic basis of bone resorption by human osteoclasts

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Abstract

Although much has been learned recently of the mechanisms that regulate osteoclastic differentiation, much less is known of the means through which their resorptive activity is controlled. This is especially so for human osteoclasts. We have developed an assay that allows us to measure resorptive activity while minimizing confounding effects on differentiation, by optimizing osteoclastogenesis, so that measurable resorption occurs over a short period, and by relating resorption in each culture during the test period to the resorption that had occurred in the same culture in a prior control period. We found that RANKL strongly stimulated release of the collagen degradation fragment CTX-I by osteoclasts over a similar range to that over which it induces osteoclastic differentiation, consistent with a distinct action on osteoclastic function. CT dose-dependently inhibited bone resorption, while PTH, IL-1, TNF- α , IL-6, IL-8, VEGF, MCP-1, MIP-1 γ , IFN- γ and dibutyryl cyclic GMP showed no significant effect. Calcium ions, cyclosporin A, IFN- β and dibutyryl cyclic AMP all strongly suppressed resorption. Bone resorption was also strongly suppressed by alendronate, the cysteine protease inhibitor E64, and the cathepsin K inhibitor MV061194. We found that inhibitors of matrix metalloproteases (MMPs) had no effect on CTX-I release. Moreover, release of the MMP-derived collagen fragment ICTP represented less than 0.01% of the quantity of CTX-I released in our cultures. This suggests that MMPs make at most a very small contribution to the bone-resorptive activity of osteoclasts.

Introduction

Calcium homeostasis, skeletal morphogenesis and bone remodeling depend on the precise regulation of the formation, localization and activity of osteoclasts, the cells that resorb bone. It is very likely that these processes are separately regulated.

However, although there have been considerable advances recently in our understanding of the mechanisms through which the differentiation of osteoclasts is regulated [1-3], much less is known of the mechanisms that modulate their function.

Human osteoclastic cells can now be generated *in vitro* by incubation of peripheral and receptor activator of nuclear factor- κ B ligand (RANKL) on plastic culture surfaces or on bone/dentine slices [4, 5]. However, although such culture systems provide powerful insights into the regulation of osteoclastic differentiation, they do not clearly distinguish between the effects of agents on differentiation and function: if osteoclasts are differentiated on bone slices, resorption is clearly dependent upon differentiation. Even those assays in which osteoclasts are generated on plastic and then suspended and sedimented onto mineralized matrix require 48h before resorption is detected [6, 7]. This time-lag implies that a differentiation step is necessary after removal from plastic. If so, this makes it very difficult to distinguish whether any increase in resorption occurs through enhanced differentiation or activation.

One potential solution to this problem has been provided by the development of assays that measure the release into culture supernatants of products of bone solubilization (CTX-I), which has been shown to correlate with bone resorption [8, 9].

If these degradation products could be measured after a short incubation period, the

contribution of differentiation to any change in resorption will be minimized. However, even in such assays, bone resorption has been measured over a period of 3 days [8, 10], so that an unknown and potentially substantial component of an observed change in resorptive activity might have been due to an effect of the test agent on differentiation rather than function. We therefore optimized osteoclastogenesis, to enable us to measure bone resorption after much shorter incubation periods, thereby minimizing confounding effects on osteoclastic differentiation. In addition, we minimized inter-culture variability by relating resorption during the test period to that during a prior control period. We have exploited this assay to analyze the humoral and pharmacological regulation of resorption by human osteoclasts.

Materials and Methods

Media and reagents

Cells were incubated in MEM with Earle's salts (Invitrogen, Paisley, UK), supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 IU/ml benzylpenicillin, and 100 µg streptomycin (all Sigma, Poole, Dorset, UK) (MEM/FCS). Recombinant human M-CSF, TNF- α , IFN- β , IFN- γ , IL-6, VEGF, MCP-1 and soluble recombinant murine RANKL were from Insight Biotechnology (Wembley, Middlesex, UK). Recombinant human IL-1 and IL-8, and recombinant murine MIP-1 γ were from R&D Systems (Abingdon, Oxfordshire, UK). The specific cathepsin K inhibitor MV061194, and the broad spectrum MMP inhibitors MV034388, BB94 and GM 6001 were respectively provided by Medivir UK (Cambridge, UK), British Biotechnology (Oxford, UK) and purchased from Merck Biosciences LH (Beeston, Nottingham, UK). Cortical bovine bone slices (4 x 4 x 0.1mm) were prepared as previously described [11]. All remaining reagents were from Sigma unless otherwise stated.

Osteoclast generation.

Heparinized blood was obtained from healthy human male or female volunteers (aged 22 - 57) with the consent of St George's Ethical Committee. The blood was layered over HISTOPAQUE-1077 and centrifuged for 30 min at 400g. The opaque interface layer of peripheral blood mononuclear cells (PBMCs) was collected with a pasteur pipette, washed in PBS and re-suspended in MEM/FCS. Cells were added (5×10^5) to the wells of 96-well plates, each well of which contained a bone slice. The cultures were incubated in a total volume of 200µl of MEM/FCS containing M-CSF (50ng/ml)

and RANKL (30ng/ml) at 37° in an atmosphere of 5% CO₂ in humidified air, for 24h. Bone slices were then removed from the wells and placed without washing in the wells of a 24-well plate, in 1ml of MEM/FCS containing M-CSF and RANKL at the same concentration, before continued incubation. Cultures were fed three times per week by replacing 60% of the medium with fresh medium with cytokines.

To monitor cultures for the development of resorptive function, representative bone slices were removed at intervals, fixed in 10% formalin and stained using toluidine blue (0.1% for 1 min). Bone slices were then inspected by light microscopy for the presence of osteoclasts and excavations. When excavation was deemed sufficient, the remaining cultures were subjected to the osteoclast resorption assay.

Osteoclast Resorption Assay

After generation of resorptive osteoclasts, bone slices were removed from the 24-well plates, washed in PBS, placed in the wells of a 96-well plate, and incubated for 24h in 100µl of fresh MEM/FCS containing M-CSF and RANKL. All the culture medium was removed after this control period of incubation and stored frozen. Bone slices were washed again in PBS and transferred to new 96-well plates containing 100µl per well of fresh MEM/FCS with M-CSF with/without RANKL, test agent or vehicle control as stated. Supernatants were collected and stored frozen after a further 24h of incubation.

The resorption activity of the cultures was determined by quantifying the C-terminal telopeptide degradation product of type I collagen (CTX-I / CrossLaps®) (Nordic Bioscience Diagnostics A/S, Herlev, Denmark) in the culture supernatants. Enzyme-

linked immunosorbent assay (ELISA) was performed according to the manufacturer's instructions. In preliminary experiments, we found no detectable CTX-I release by PBMC-derived cells incubated on plastic in the presence of RANKL, and CTX-I release was undetectable from bone slices incubated in culture medium without cells. We also measured matrix metalloprotease (MMP)-mediated degradation of collagen in some supernatants by quantifying the concentration of carboxyterminal cross-linked telopeptide of type I collagen (ICTP), using Orion Diagnostic UniQ ICTP enzyme-immunoassay (Oxford Biosystems, Oxford, UK), according to the manufacturer's protocol.

In some experiments, when the resorption assay had been completed, bone slices were further analyzed for visualization of cells and excavations by staining with 0.1% toluidine blue or for TRAP using the Leucognost-AP cytochemical reagent kit (VWR International Ltd, Lutterworth, Leicester, UK) after fixation in 10% formalin, or by scanning electron microscopy.

Scanning Electron Microscopy

After incubation, bone slices were immersed in 10% (v/v) sodium hypochlorite for 10 min to remove cells. The bone slices were then washed in water, air-dried, mounted onto stubs, sputter-coated with gold and inspected in a scanning electron microscope (S90: Cambridge Instruments, Cambridge, UK)

Statistical Analysis

All data are expressed as mean $\pm$ standard error of mean, of four replicate cultures, unless otherwise stated. The significance of differences between control and

experimental groups was assessed by ANOVA (Bonferroni's Post Test). Differences were considered significant if $p < 0.05$.

Results

Our experience is that, with carefully selected combinations of serum batch and donor, substantial numbers of osteoclasts form on bone slices and show extensive bone resorption within 7 days (Fig 1). This extensive resorption was reflected in substantial CTX-I release. The resorption that can be achieved by this approach exceeds that reported in previous publications [8, 10] by at least an order of magnitude. This enabled measurement of resorption over a substantially shorter time than has previously been used (24 hours, versus 3 days). This minimizes the confounding effect of agents on osteoclast differentiation, and facilitates the identification of the separate effect of agents on bone resorption. The assay also minimizes inter-culture variability in bone resorption, by relating bone resorption during the test period to that of the same culture during the control period.

RANKL strongly stimulated CTX-I release by already-formed osteoclasts (Fig 2A) over a similar range to that which induced differentiation of osteoclasts (Fig 2B). Since osteoclasts were already formed before resorption was measured, this result suggests that RANKL not only induces osteoclastic differentiation, but also has a distinct action whereby it stimulates resorption by existing osteoclasts. The increase in CTX-I release induced by RANKL is similar to the increase in the area of bone surface resorbed by *ex vivo* rodent osteoclasts [12, 13].

CT suppressed RANKL-stimulated bone resorption to the levels seen in M-CSF alone (Fig 3), while PTH showed no effect on resorption, either in cultures incubated in M-

CSF and RANKL, or in M-CSF alone. Neither IL-1, MIP-1 γ nor TNF- α stimulated resorption in cultures to which RANKL was not added.

Addition of OPG to cultures did not decrease resorption compared to cultures incubated in M-CSF alone (Fig 4). This suggests that the residual resorption observed in such cultures is not due to residual RANKL, but rather that RANKL induces a state of activation that persists for some time, even in the absence of RANKL. *Ex vivo* osteoclasts show a similar behavior, since they resorb bone despite physical isolation and lack of exogenous RANKL [12, 13]. Calcium ions, cyclosporin A and IFN- β all strongly suppressed RANKL-stimulated resorption. Dibutyryl cyclic AMP suppressed resorption to below levels observed in M-CSF alone, suggesting that this agent is a potent inhibitor of the pathways that lead to or maintain activation of osteoclasts by RANKL. In contrast, dibutyryl cyclic GMP was without effect on bone resorption, as was IL-8.

VEGF and MCP-1 showed no effect on either basal or RANKL-stimulated resorption (Fig 4). We found that IL-6 did not stimulate resorption in M-CSF-incubated cultures; and IFN- γ did not suppress RANKL-stimulated bone resorption.

Human calcitonin (CT) dose-dependently inhibited bone resorption, with significant inhibition occurring at 1ng/ml (Fig 5). Bone resorption was also strongly suppressed by alendronate, the general cysteine protease inhibitor E64, and MV061194, a specific inhibitor of cathepsin K.

It has been suggested that MMPs contribute to bone resorption by osteoclasts. We found that the broad-spectrum inhibitors of matrix metalloproteases BB94, MV034388 and GM6001 had no effect on CTX-I release (Fig 6). To determine whether this lack of detectable effect was due to an inhibitory effect that was too small to detect against the variance of the large baseline of resorption, we also tested the ability of GM6001 to suppress CTX-I release in cultures in which cathepsin K was inhibited. We found (Fig 6), even against this smaller baseline, no significant further inhibition by the MMP inhibitor GM6001.

Because there is uncertainty over whether MMPs do [14] or do not [15] release CTX-I from collagen, we also measured the concentration in supernatants of the ICTP antigen, which is derived from MMP-mediated degradation of collagen. We found (Fig 7) that ICTP release was very small indeed compared with CTX-I release (assuming that ICTP is 10.4kD, then ICTP release is approximately 200pM, while CTX-I release is approximately 1 μ M in the same cultures; ie ICTP release in the control 'basal' period represents approximately 1/5000 of the CTX-I release in the same period). This suggests that MMPs make at most a very small contribution to the bone-resorptive activity of osteoclasts. We also noted that this ICTP release was not increased by RANKL, suggesting that this release might not reflect the same process as bone resorption, which is induced by RANKL. ICTP concentration was increased by approximately 40% in the presence of the cathepsin K inhibitor. This is consistent with the known ability of cathepsin K to degrade the ICTP antigen.

Discussion

Osteoclasts show morphological evidence *in vivo* of changes in functional activity that correspond to changes in plasma calcium concentrations very shortly after administration of hormones such as CT or PTH [16, 17]. Only much later do osteoclast numbers change. Thus, modulation of the activity of existing osteoclasts is a major component of the regulation of bone resorption. Recently, significant advances have been made in elucidating the mechanisms that govern osteoclastogenesis [1, 3, 18]. In contrast, much less is known about how the resorptive activity of osteoclasts is regulated.

The major obstacle has been that osteoclastic resorption depends on osteoclastic differentiation, and consequently it cannot be readily determined whether an increase in resorption has occurred through enhanced differentiation, or through increased resorption by osteoclasts already formed. The development of assays that measure release of collagen degradation products into the culture medium provides a potential solution to these problems: if such products can be detected after a short incubation period, the contribution of differentiation to any increase in resorption can be minimized.

Previous reports have measured release over 3-day culture periods [8, 10]. This provides ample time for effects on differentiation to interfere with the interpretation of the data. We have found that, by optimizing the culture conditions, we can readily detect collagen degradation products released into the supernatant after 24h of incubation. This short incubation period minimizes the confounding effect of agents

on osteoclast differentiation, and facilitates the identification of the separate effect of agents on bone resorption. In addition, we minimized inter-culture variability by relating bone resorption during the test period to that during the control period. We exploited this assay to analyze the humoral and pharmacological regulation of resorption by human osteoclasts.

We noted that several agents that stimulate bone resorption in intact bone had no effect on osteoblast-free cultures. PTH, IL-6, VEGF, TNF- α and MCP-1, all of which are considered to have a role in the regulation of bone resorption, were without effect. The inability of TNF- α or IL-1 to stimulate the function of PBMC-derived human osteoclasts directly contrasts with the potent ability of both cytokines to stimulate osteoclastic function in bone marrow-derived murine osteoclasts [19-21]. TNF- α has been shown to induce osteoclast formation in human monocytes [22], as it does in murine bone marrow cells [19, 23].

The IFNs are potent inhibitors of osteoclastic differentiation [24, 25], yet resorption was only partially inhibited by IFN- γ . This suggests that the effect of IFN- γ on osteoclastic differentiation may be primarily a consequence of its role in the regulation of macrophages. The results also emphasize that differentiation and activation are separately regulated by distinct agents.

It has recently been found that activation of NFAT is necessary and sufficient for osteoclast formation [26, 27]. We found that cyclosporin A, which inhibits the activation of NFAT, strongly inhibits bone resorption. This suggests that activation of NFAT is necessary for both osteoclast formation and function. RANKL has been

shown to induce intracellular calcium oscillations, which are presumed to be responsible for the activation of NFAT [26]. High extracellular concentrations of calcium are known to increase intracellular calcium through a putative osteoclastic calcium sensor [28, 29], yet bone resorption is suppressed by high extracellular calcium concentrations in human as in murine [30] osteoclasts. Whether increased intracellular calcium enhances osteoclastic differentiation or suppresses osteoclastic function may depend upon its localization, pulsatility and level [31].

We found that the resorptive activity of human osteoclasts was potently suppressed by CT, alendronate, the broad-specificity cysteine protease inhibitor E64 and the cathepsin K-specific inhibitor MV061194. The last results are consistent with the observation that deletion of the gene for cathepsin K, the predominant cathepsin expressed by osteoclasts, or mutation in humans of the same gene in pycnodysostosis, causes osteopetrosis. It has however been noted that genetic deficiency of cathepsin K is associated with an increase in serum levels of the ICTP antigen [32, 33], which is generated from collagen by MMPs. This has led to the suggestion that MMP-mediated bone matrix solubilization can compensate for cathepsin K deficiency [32].

The above considerations potentially undermine the reliability of CTX release as a measure of matrix degradation by osteoclasts in the presence of protease inhibitors. Therefore, we measured the effect of MMP inhibitors in our assay. Whether the CTX-I assay does [14] or does not [15] detect MMP products, MMPs have been shown to increase the amount of CTX released from collagen by cathepsin K [15]. Thus, if osteoclastic MMPs contribute to bone resorption, we would expect a decrease in CTX-I by the MMP inhibitors. We found no detectable suppression. To further assess

the contribution of MMPs to bone resorption, we measured release of the MMP-derived ICTP fragment of collagen by osteoclasts. We found that, even in the presence of a cathepsin K inhibitor (to inhibit degradation of ICTP by cathepsin K [14, 34, 35], ICTP release was very small compared with CTX-I release (approximately 1/5000 of the rate of resorption in the control period), which is quantitatively inadequate to represent physiologically-significant compensation. We also noted that there was a 40% increase in ICTP release by osteoclasts in the presence vs absence of cathepsin K inhibitor, which is a similar increase to that (50%) observed *in vivo* in genetic deficiency of cathepsin K [32, 33]. These results are consistent with the notion that the increase in ICTP *in vivo* in pycnodysostosis can be explained by absence of cathepsin K-mediated ICTP degradation.

It thus appears that bone resorption by osteoclasts is overwhelmingly cathepsin K-dependent. This does not imply that MMPs play no role in bone resorption. There is good evidence that not only cathepsins, but MMPs are essential for normal bone resorption in organ-cultured bone, and that both osteoblastic cells (bone lining cells and osteocytes) and osteoclasts express MMPs. Osteoclastic MMPs have been shown to play a role in migration [36]. Osteoblastic MMPs might participate in bone resorption *in vivo* by removal of the osteoclast-inhibitory layer of organic material that lines bone surfaces, and/or might participate by debridement of demineralized matrix from osteoclastic excavations. Bone resorption is only partially impaired by cathepsin K deficiency *in vivo*, yet essentially abrogated *in vitro*. It may be that compensation for cathepsin K deficiency occurs *in vivo* through episodic removal of osteoclast-demineralised collagen by osteoblastic cells. Whether or not this is so, it

appears that the assay we have described provides a reliable means to measure the regulation of the resorptive function of osteoclasts.

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Figure Legends

Figure 1. Osteoclast formation and bone resorption in cultures of human PBMCs. PBMCs were incubated on bone slices in M-CSF (50ng/ml) and RANKL (30ng/ml) for 7 days before they were stained with toluidine blue (A,B) to assess multinuclear cell formation and bone resorption (bar = 100µm). In A, the bone slice is covered by many very large cells, with only very occasional remaining small (mononuclear) cells (arrowheads). Similarly rare mononuclear cells are shown amongst large cells in B (arrowheads). B: at higher magnification excavations (arrows) can be visualized as darkly-staining areas beneath cells. C: SEM of bone slice after removal of cells. Almost the entire surface shows excavation, with only occasional islands of unresorbed surface remaining.

Figure 2. Comparison between the ability of RANKL to stimulate resorption in already-formed osteoclasts, and its ability to induce osteoclastic differentiation. A: PBMCs were incubated on bone slices for 7 days in M-CSF (50 ng/ml) and RANKL (30 ng/ml). After 7 days, bone slices with osteoclasts were transferred to new wells with fresh M-CSF and RANKL, and cells incubated for 24h. Supernatant was then collected for CTX-I assay, and bone slices were transferred to fresh wells with medium containing M-CSF and RANKL (0-30ng/ml). Supernatants were collected after a further 24h for CTX-I assay. Resorption in second period was expressed as the percentage of that in the same culture in the first period. CTX-I concentrations after basal, control period, for 0, 1, 3, 10, and 30 ng/ml RANKL were (group means): 1060, 620, 721, 625, and 439 nM respectively. B: PBMCs were incubated in M-CSF with/without the shown concentration of RANKL for 9 days. After incubation, cells

were reacted for TRAP, and the number of TRAP-positive cells with 3 or more nuclei quantified by light microscopy. n=4 cultures per variable. *p<0.05 vs controls (ANOVA) vs no RANKL.

Figure 3. Effect of CT (1ng/ml), PTH (0.1U/ml), IL-1 (10ng/ml), MIP-1 γ (500ng/ml) and TNF- α (10ng/ml) on CTX-I release by PBMC-derived human osteoclasts.

PBMCs were incubated on bone slices for 7 days, before incubation in fresh medium with M-CSF and RANKL for 24h, followed by incubation in M-CSF with or without the cytokines shown for 24h. CTX-I concentrations in the basal period ranged between (group means) 87-253 nM. * p<0.05 vs no RANKL; a: p<0.05 vs RANKL (ANOVA). RL=RANKL

Figure 4. Effect of OPG (100ng/ml), calcium (20mM), cyclosporin A (10 μ g/ml), IFN- β (20ng/ml), dibutyryl cyclic AMP (10⁻³M), dibutyryl cyclic GMP (10⁻³M), IL-8 (20ng/ml), VEGF (100ng/ml), MCP-1 (100ng/ml), IL-6 (20ng/ml) and IFN- γ (20ng/ml) on CTX-I release by PBMC-derived human osteoclasts. PBMCs were incubated on bone slices for 7 days, before incubation in fresh medium with M-CSF and RANKL for 24h, followed by incubation in M-CSF with or without the cytokines shown for 24h. CTX-I concentrations (group means) after the basal period ranged between (nM) A: 29-75; B: 978-1321. * p<0.01 vs no cytokines; a: p<0.01 vs RANKL (ANOVA).

Figure 5. Effect of CT, alendronate, E64 and MV061194 on CTX-I release by PBMC-derived human osteoclasts. PBMCs were incubated on bone slices for 7 days, before incubation in fresh medium with M-CSF and RANKL for 24h, followed by

incubation in the agents shown for 24h. All test period incubations were performed in the presence of M-CSF and RANKL. CTX-I concentrations after the basal period ranged between (group means) (nM) 55-185 (A); 563-698 (B); 976-1440 (C; 647-1032 (D). * $p < 0.05$ vs controls (ANOVA).

Figure 6. Effect of MMP-inhibitors MV034388 (10^{-6} M), BB94 (10^{-6} M), and GM6001 (1.3×10^{-5} M), with and without cathepsin K inhibitor MV061194 (3×10^{-7} M) on CTX-I release by PBMC-derived human osteoclasts. PBMCs were incubated on bone slices for 7 days, before incubation in fresh medium for 24h, followed by incubation in the agents shown for 24h. All incubations were in the presence of M-CSF and RANKL. Mean CTX-I concentrations of groups after the basal period ranged between (nM) 172-423 (A); 796-951 (B). * $p < 0.05$ vs control (ANOVA).

Figure 7. Effect of MMP-inhibitor GM6001 (1.3×10^{-5} M) and cathepsin K inhibitor MV061194 (3×10^{-7} M) on release of CTX-I and ICTP by PBMC-derived human osteoclasts. PBMCs were incubated on bone slices for 7 days, before incubation in fresh medium for 24h, followed by incubation in the agents shown for 24h. All incubations were performed in the presence of M-CSF and RANKL. Results shown as release after test period expressed as percentage of that released during the previous control 'basal' period. ICTP concentrations after the basal period were ($\mu\text{g/ml}$) (left to right)(group means): 1.62, 2.06, 1.86, 2.37, 2.43. CTX-I concentrations after the basal period were (nM) (left to right) (group means): 1328, 952, 1279, 961, 1119. * $p < 0.01$ vs no RANKL; a: $p < 0.05$ vs RANKL alone (ANOVA).

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