Metabolic activation stimulates acid secretion and expression of matrix degrading proteases in human osteoblasts

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Background: Both cellular and matrix components of healthy bone are permanently renewed in a balanced homeostasis. Osteoclastic bone resorption involves the expression of vacuolar-type ATPase proton pumps (vATPase) on the outer cell membrane and the secretion of matrix degrading proteases. Osteoblasts modulate the deposition of bone mineral components and secrete extracellular matrix proteins.

Objectives: To investigate the ability of osteoblasts and osteosarcoma cells to secrete acid and express matrix degrading proteases upon metabolic activation. To examine also the potential contribution of vATPases to proton secretion expressed on osteoblasts.

Methods: Osteoblasts were isolated from trabecular bone and characterised by reverse transcriptase-polymerase chain reaction and immunohistochemistry. Proton secretion was analysed by a cytosensor microphysiometer.

Results: Osteoblasts not only express matrix degrading proteases upon stimulation with tumour necrosis factor or with phorbol ester but they also secrete protons upon activation. Proton secretion by osteoblasts is associated partially with proton pump ATPases.

Conclusion: These data suggest that, in addition to monocyte derived osteoclasts, cytokine activated mesenchymal osteoblasts and osteosarcoma cells may contribute to the acidic milieu required for bone degradation.

B one tissue is constantly in a process of remodelling. For bone degradation, osteoclasts express matrix degrading proteases, including different cathepsins and matrix metalloproteinases (MMPs).

Further, osteoclasts secrete protons by a vacuolar (H+/ATPase (vATPase)). Mutations of a vATPase subunit cause osteopetrosis and deletion of a vATPase subunit results in osteosclerosis.

Previously the vATPase activity was associated with osteoclasts only. But recently, vATPases have been described in a variety of other cell lineages as well. Such vATPases act in intracellular pH regulation as well as in proton secretion. This prompted us to study proton secretion by osteoblasts.

Parathyroid hormone has been shown to induce proton secretion by osteoclasts or osteosarcoma cells. We have shown that synovial fibroblasts secrete protons upon metabolic activation, and fibroblasts isolated from the synovial-like interface membrane can resorb bone without the help of osteoclasts. These results suggested that activated mesenchymal cells secrete considerable amounts of protons and thereby contribute to bone degradation under certain conditions. As osteoblasts are closely related to fibroblasts, we investigated osteoblasts as a second proton source in bone besides osteoclasts.

Protons have an important role in collagen degradation of demineralised bone areas or in cartilage as an acidic milieu is a prerequisite for optimal enzymatic activity of collagenolytic enzymes such as cathepsins, and also for solubilisation of collagen fibres before enzymatic degradation by collagenases. Consequently, enhanced proton secretion contributes to degeneration of articular cartilage in rheumatoid arthritis and in other pathological conditions of the musculoskeletal apparatus as well. Therefore, we investigated mechanisms of proton secretion in human osteoblasts.

MATERIALS AND METHODS

Preparation of osteoblasts

Osteoblasts were isolated from trabecular bone and expanded in Dulbecco’s modified Eagle’s medium enriched with 20% fetal calf serum and antibiotics. Osteosarcoma lines SAOS-2 and MG-63 (ATCC) served as osteoblast controls. The cells were characterised by reverse transcriptase-polymerase chain reaction (RT-PCR; table W1 (available at http://www.annrheumdis.com/supplemental)) and immunohistochemistry. Secretion of MMPs was evaluated by enzyme linked immunosorbent assay (ELISA).

Cytosensor microphysiometer analysis

Proton secretion was analysed by a cytosensor microphysiometer, and pericellular acidification was measured as described previously. In brief, proton release of the cells after stimulation increases the acidification rate (fig W1). For induction experiments cells were stimulated with tumour necrosis factor α.

Abbreviations: BMP, bone morphogenic protein; MMP, matrix metalloproteinase; PMA, phorbol myristate acetate; RT-PCR, reverse transcriptase-polymerase chain reaction; TNFα, tumour necrosis factor α.
factor α (TNF; 1–100 ng/ml) or phorbol myristate acetate (PMA; 0.1 ng/ml to 10 μg/ml). To block Na+/H+ transmembrane proton pumps or vacuolar-type H+ ATPases (vATPases), Amiloride (0.5 and 1 mmol/l) or Bafilomycin A1 (1 and 2 μmol/l; Calbiochem, Bad Soden, Germany) were added to the osteoblasts, and proton secretion was recorded.

RESULTS

Functional characterisation of the osteoblasts

To characterise the cells under study, the expression of osteocalcin was detected by immunohistochemistry (fig W2A (http://www.annrheumdis.com supplemental)), and osteoblast associated genes, including alkaline phosphatase, bone morphogenic protein (BMP)-2, BMP-4, osteocalcin, and osteopontin, were detected by RT-PCR analysis, confirming that the cells under investigation were osteoblast-like cells (fig W2B (http://www.annrheumdis.com supplemental)).

Proton secretion by osteoblasts

Phorbol ester (PMA) activates protein kinase C and parathyroid hormone induced pericellular acidification in SAOS-2 osteosarcoma by a protein kinase C dependent pathway. Consequently, we tested osteoblasts and osteosarcoma cells for proton secretion upon activation with PMA. In early passage osteoblasts (passage 4–8), PMA induced a dose dependent acidification, and 35% proton secretion above equilibrium was reached (fig W3A (http://www.annrheumdis.com supplemental)). After long term culture (10–12 passages) osteoblasts responded with lower sensitivity to PMA induced proton secretion (fig W3A (http://www.annrheumdis.com supplemental)). Further, time course experiments showed that proton secretion was activated immediately after addition of PMA, reaching the plateau as early as 10 minutes after induction (fig 1A). PMA-induced proton secretion in SAOS-2 osteosarcoma cells confirmed that mesenchymal cells secrete protons upon metabolic activation, and a proton secretion above 40% was induced with PMA (fig W3A (http://www.annrheumdis.com supplemental)).

TNFα stimulates proton secretion in osteoblasts

We next tested TNFα for its induction of proton secretion by osteoblasts. Early passage osteoblasts responded to TNFα at higher concentrations (10–100 ng/ml) with a proton secretion of 20–35% above the equilibrium level. Low concentrations of TNF (1–2 ng/ml) induced an acidification rate of about 20% above the equilibrium level. The TNF-induced proton secretion was reduced in late passage osteoblasts (fig W3B (http://www.annrheumdis.com supplemental)). Further, time course experiments showed that the TNF response was delayed to some extent (fig 1B) in comparison with the PMA induced acidification (fig 1A), and the maximal proton secretion was reached 20 minutes after induction (fig 1B).

As different mechanisms may contribute to pericellular acidification, we used Bafilomycin A1, a specific vATPase blocker (1 μmol/l, 2 μmol/l), and Amiloride, a blocker of Na+/H+ exchange ATPases at a higher concentration (500 μmol/l, 1 mmol/l), to reduce the proton secretion. Both compounds reduced proton secretion by 5 and 12% or by 10 and 12%, respectively (fig 2). This indicates that the involvement of proton pump ATPases of either the vacuolar type (vATPase) or Na+/H+ exchange ATPases works across the extracellular membrane of osteoblasts, thus contributing to the acidification reported here.

Expression of proteolytic enzymes in activated osteoblasts

To test whether activated osteoblasts may contribute to degradation of extracellular matrix proteins, the expression of proteolytic enzymes was investigated. In osteoblasts mRNA encoding MMP-1 was enhanced by addition of TNF (p<0.17) and PMA (p<0.17) about 25-fold (fig W4A (http://www.annrheumdis.com supplemental)). MMP-3 encoding mRNA was stimulated by TNF (p<0.136) and PMA (p<0.05) on average fivefold as determined by real time quantitative RT-PCR (fig W4A). Further, TNF augmented MMP-1 (p<0.13) and MMP-3 (p<0.02) concentrations in osteoblast supernatants. Comparedly, PMA stimulated

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**Figure 1**

Activation of proton secretion (r_max/r_eq in per cent) in osteoblasts upon stimulation by PMA or TNF. (A) Osteoblasts were preincubated for about 20 minutes in running medium to reach metabolic quiescence characterised by a low spontaneous acidification (r(t)=r_eq, white zone). After 15 minutes of preincubation the fluctuations of the acidification value drop below 5%. Then cells are activated with PMA (grey zone), and the metabolic responses are recorded as a function of time. Each data point represents the mean value of n individual measurements of r_max (n=5–10) and the error bars represent the normalised standard deviations. Addition of 1 μg/ml PMA induced an immediate increase in acidification, reaching a plateau at about 115% after 20 minutes. Addition of 5 μg/ml PMA induced a higher response reaching more than 120% acidification. Addition of 10 μg/ml PMA induced an immediate acidification response and a plateau was not observed after 40 minutes of induction. (B) Osteoblasts were activated with TNF (grey zone) as described above (see (A)). Addition of 20 ng/ml TNF induced a slow increase in acidification reaching about 115% after 20 minutes. Addition of 50 ng/ml TNF induced a response reaching more than 120% acidification. Addition of 100 ng/ml TNFα at first reduced the acidification rate below the equilibrium level, then an acidification response was recorded reaching about 125% after 20 minutes.

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metabolism. Amiloride, reduced proton secretion by 5–12%. Metabolic activation in human osteoblasts 69 + Bafilomycin A1, and the blocker of Na+/H+ transmembrane exchange pathways, Amiloride, reduced proton secretion by 5–12%.

**DISCUSSION**

Previously, we showed that fibroblasts may secrete protons upon metabolic activation. To study osteoblast associated proton secretion we activated the cells by addition of PMA, as this compound has previously been associated with proton secretion in bone and with pericellular acidification through osteoblasts. T cells, or peritoneal macrophages.

Interestingly, an overall trend towards a lower acidification response was noted in late passage osteoblasts in comparison with early passage cells or SAOS-2 in osteosarcoma cells. However, detection of osteoblast marker genes supports our suggestion that besides osteoclasts the osteoblasts may secrete protons upon activation. Addition of TNF was used as a physiological activator of the osteoblasts. TNF is a prominent product of monocytes and, in addition, TNF activates osteoclast resorptive activity. In our experiments, TNF activated proton secretion in human normal osteoblasts.

Activated osteoblasts express different matrix MMPs. The activation of proton secretion in the presence of enhanced MMP activity may result in a catabolic situation. Our data support these findings as expression of MMP-1 and MMP-3 were up regulated in osteoblasts. However, owing to variations in gene induction in the individual samples the statistical significance was not high. Nevertheless, induction of MMP-1 and MMP-3 were recorded in all individual experiments.

The resorptive capacity of cells in bone is principally associated with osteoclasts or tumour associated macrophages. Proton secretion by individual osteoclasts is probably rather small as they lack ruffled membrane areas equipped with proton pumps. Of note, osteoclasts outnumber osteoblasts in bone. The lifespan of osteoclasts and their differentiation capabilities are limited at least in vitro in comparison with osteoblasts. In addition, osteoclasts are rather sensitive to apoptosis inducing signals in comparison with osteoblasts. Although osteolysis by tumours was shown to require osteoclasts in vivo, osteoclasts or osteosarcomas may represent a considerable proton source in bone under specific conditions. Consequently, for physiological bone turnover processes such as growth of bone, wound healing, or load adaptation, osteoclasts represent the primary cells for mineral and protein matrix degradation. In chronic inflammatory processes, osteoporosis of the elderly, cancer, or under other pathological conditions the slow resorbing but apoptosis resistant osteoblast may contribute to osteolysis.

The specific contribution of different cellular proton sources for pericellular acidification by osteoblasts remains to be investigated. Enhanced glycolysis may account for pericellular acidification. This may explain in part the observation that early passage osteoblasts show higher proton secretion than the same cells at later passages or SAOS-2 cells. However, as cells are serum starved before cytosensor microphysiometry, glycolysis is probably considerably reduced under these conditions. This indicates that additional proton sources are active on osteoblasts. Such proton sources include Na+/H+ ion exchange ATPases or V-ATPases. As we found mRNA encoding the 116 kDa subunit of the V-ATPase in osteoblasts (not shown) and a reduced pericellular acidification upon addition of the specific proton pump blocker Bafilomycin A1 and Amiloride to the cells, our data suggest that specific cell membrane associated ATPases participate in proton extrusion and in pericellular acidification by osteoblasts or osteosarcoma cells. Therefore, mesenchymal cells may contribute directly to tissue degradation using different proton sources, including V-ATPase and H+ ion exchange ATPase activities and matrix degrading proteases. To the best of our knowledge, this is the first report of mechanisms of pericellular acidification by proton pumps on osteoblasts or osteosarcoma cells.

**ACKNOWLEDGEMENTS**

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**REFERENCES**


BOOK REVIEW

Osteoarthritis. 2nd ed

The second edition of Osteoarthritis is a very well documented and illustrated book. The different chapters cover perfectly the most important questions relating to osteoarthritis—epidemiology, socioeconomics, genetics, pathogenesis, clinical features, and management both in daily practice and clinical trials. In particular, there are well documented sections on specific investigations such as biological markers, magnetic resonance imaging, and ultrasonography. Moreover, two sections evaluate more fully the potential modifiers of cartilage breakdown. This book will be useful for all who are involved in osteoarthritis research and/or in charge of the management of osteoarthritis patients.

M Dougados

FORTHCOMING EVENTS

International Congress on SLE and Related Conditions
9–13 May 2004; New York, New York, USA
Contact: The Oakley Group, 2014 Broadway, Suite 250, Nashville, Tennessee 37203, USA

4th International Congress on Autoimmunity
3–7 November, 2004; Budapest, Hungary
Deadline for receipt of abstracts: 20 June 2004
Contact: 4th International Congress on Autoimmunity, Kenes International—Global Congress Organisers and Association Management Services, 17 rue du Cendrier, PO Box 1726, CH-1211 Geneva 1, Switzerland
Tel: +41 22 908 0488
Fax: +41 22 732 2890
Email: autoim04@kenes.com
Website: www.kenes.com/autoim2004

CORRECTION

Corrections printed in the journal also appear on the Annals website www.annrheumdis.com and are linked to the original publication doi: 10.1136/ard.2002.005256corr1


We regret that in fig 1B of this article the lines joining the data points did not appear in the shaded area of the figure. The correct figure is shown below.

Figure 1  Activation of proton secretion in osteoblasts upon stimulation by PMA or TNF.

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The cytosensor microphysiometer

Osteoblasts are grown on a semipermeable membrane in a cup placed on top of a sensor chip. The photocurrent $I(U)$ is recorded between the reference electrode in the medium and the back of the sensor chip while the bias voltage $U$ is periodically changed by a connected computer. Proton secretion induces changes in $I(U)$. 
Analysis of Gene Expression Patterns in Osteoblasts

Bone cells were expanded and characterised by immunohistochemistry to confirm their osteoblastic lineage. The cells were stained with anti-human osteocalcin serum followed by detection reagents (left) or with detection reagent only omitting the specific antibody (right).
Figure W2B.
MWM

GAPDH

AP

BMP-2

BMP-4

OCN

OPN
Analysis of Gene Expression Patterns in Osteoblasts

Additional osteoblastic marker genes were detected by RT-PCR analysis and mRNA encoding alkaline phosphatase (AP), bone morphogenetic protein (BMP)–2 and BMP–4, osteocalcin (OCN) and osteopontin (OPN) were detected in all three osteoblast samples (lanes 2 – 4 of each gel). GAPDH RT-PCR served as external control (top), the molecular weight marker (MWM, 100 bp ladder) is shown on the left of each gel.
Activation of proton secretion ($r_{max}/r_{eq}$ in %) in early and late passage osteoblasts and in SAOS-2 osteosarcoma cells upon stimulation by PMA or TNF-α.

Cells were activated with PMA at the concentrations shown and the metabolic activation was recorded by a cytosensor microphysiometer (early passage osteoblasts: black squares; late passage osteoblasts: open squares; SAOS-2 osteosarcoma: black circles). The amount of 100% acidification represents the equilibrium value for proton secretion of the cells without stimulation. Each data point represents the mean value $x_i$ of $n$ individual measurements (early passage osteoblasts: $n = 3$ or $4$; late passage osteoblasts: $n = 2$ to $5$; SAOS-2: $n = 5$ to $10$) and the error bars represent the normalized standard deviations of $x_i$. Overall, late passage osteoblasts show little acidification rates when compared to early passage osteoblasts or osteosarcoma cells. Because of procedural variability in determining
the $r(t)/r_{eq}$ rates, only changes in acidification rates exceeding 5% are regarded as a sizeable change.
Induction of matrix degrading proteases in osteoblast-like cells

Osteoblasts were activated by addition of 50 ng/ml TNF-α or 1 ng/ml PMA for 24 h and transcripts encoding MMP-1 (top) MMP-3 (bottom) were enumerated by real time quantitative RT-PCR in comparison to mock treated controls (A). The data show the mean values and standard errors of induction experiments using osteoblasts from three individuals separately. We present the target gene to GAPDH signal ratio (Y-axis, induction index over control) as function of the induction reagent (x-axis). In all samples analysed, activation of osteoblasts resulted in enhanced steady state mRNA levels encoding MMP-1 or MMP-3 (A). In addition, the supernatants were harvested
and concentrations of MMP-1 (top) or MMP-3 (bottom) were enumerated by ELISA in comparison to the controls (B). The figures represent the mean values and standard errors of two independent duplicate induction experiments using osteoblasts from three individuals separately. Dilutions of recombinant collagenases served as positive controls, complete medium as negative control. The scale (Y-axis) shows the MMP-1 or MMP-3 concentrations in ng/ml. In all samples analysed, activation of the cells by TNF-α or PMA resulted in enhanced MMP-1 and MMP-3 secretion (B).
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Table W1:

Oligonucleotide primers for RT-PCR Experiments

<table>
<thead>
<tr>
<th>Gene</th>
<th>Oligo product (pb)</th>
<th>Oligo</th>
<th>Oligo</th>
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<tr>
<td>Alk. Phos.</td>
<td>454</td>
<td>5'TGGAGCTTCAGAAGCTCAACACCA</td>
<td>5'ATCTCGTTGTCTGAGTACCAGTCC</td>
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<tr>
<td>BMP-2</td>
<td>671</td>
<td>5'GCTGTACTAGCGACACCCAC</td>
<td>5'TCATAAAACCTGCAACAGCCAACTCG</td>
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<tr>
<td>BMP-4</td>
<td>346</td>
<td>5'GCTGAAGTCCACATAGAGCGAGTG</td>
<td>5'ACTGGTCCACCACAATGTGACACG</td>
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<tr>
<td>osteopontin</td>
<td>492</td>
<td>5'AGCCGTGGGAAGGACAGTTA</td>
<td>5'TGAAGCTTTTAGTTTACAGGGAGTTT</td>
</tr>
</tbody>
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