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 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.

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 (http://www.annrheumdis.com/supplemental)). For induction experiments cells were stimulated with 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. Consequentially, 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<0.017) and PMA (p<0.017) 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. Comparably, PMA stimulated

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 x_i (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 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)).


Figure 2  Blocking proton secretion by osteoblasts upon addition of Bafilomycin A1 or Amiloride. Osteoblast-like cells were incubated in medium containing 1 mM and 2 mM Bafilomycin A1 or 500 mM and 10000 mM Amiloride. Pericellular acidification was recorded by cytosensor microphysiometer. Each data point represents the mean of two individual measurements and the error bars represent the normalised standard deviations. Both the vATPase proton pump blocker, Bafilomycin A1, and the blocker of Na\(^+\)/H\(^+\) transmembrane exchange ATPases or H\(^+\) ion exchange ATPases, respectively, 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 vATPase 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.

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REFERENCES


Limited space in printed journals means that interesting data and other material are often edited out of articles; however, limitless cyberspace means that we can include this information online.

Look out for additional tables, references, illustrations.

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**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 osteoarthritic patients.

M Dougados

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**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

**IOF World Congress on Osteoporosis**
14–18 May 2004; Rio de Janeiro, Brazil
IOF awards are available for scientists:
IOF Claus Christiansen Research Fellowship: 45 000
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Contact: Congress Secretariat at info@osteofound.org
Website: www.osteofound.org

**International Society for the Study of the Lumbar Spine**
31 May–3 June 2004; Porto, Portugal
Contact: International Society for the Study of the Lumbar Spine, 2075 Bayview Avenue, Room MG 323, Toronto, Ontario, Canada M4N 3M5
Tel: 00 1 416 480 4833
Fax: 00 1 416 480 6053
Email: shirley.fitgerald@sw.ca

**Xth International Conference on Behçet’s Disease**
27–31 October 2004; Antalya, Turkey
Contact: Congress Secretariat, Figur Congress and Organization Services Ltd. STL, Ayazmaderesi Cad. Karadut Sok. No: 7 80888 Dikilitas, Istanbul, Turkey
Tel: +90 (0212) 258 6020
Fax: +90 (0212) 258 6078
Email: behcet2004@figur.net
Website: www.behcet2004.org

**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 2850
Email: autoim04@kenes.com
Website: www.kenes.com/autoim2004

**VIIth European Lupus Meeting**
3–5 March 2005; Royal College of Physicians, London, UK
Contact: Julia Kermode, Conference organiser of the British Society of Rheumatology
Email: Julia@Rheumatology.org.uk

**Future EULAR congresses**
9–12 June 2004; EULAR 2004; Berlin, Germany
8–11 June 2005; EULAR 2005; Vienna, Austria
21–24 June 2006; EULAR 2006; Amsterdam, The Netherlands

**Future ACR meeting**
16–21 October 2004; 68th Annual Scientific Meeting; San Antonio, Texas

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**CORRECTION**

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 TNFα.

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*PostScript*