Research article

Osteoclast-independent bone resorption by fibroblast-like cells

Thomas Pap[1,2], Anja Claus[3], Susumu Ohtsu[2], Klaus M Hummel[3], Peter Schwartz[4],
Susanne Drynda[1], Géza Pap[5], Andreas Machner[5], Bernhard Stein[6], Michael George[6],
Renate E Gay[2], Wolfram Neumann[5], Steffen Gay[2] and Wilhelm K Aicher[7]

[1]Division of Experimental Rheumatology, Otto-von-Guericke University, Magdeburg, Germany
[2]Center of Experimental Rheumatology, Department of Rheumatology, University Hospital, Zurich, Switzerland
[3]Division of Nephrology and Rheumatology, Georg-August-University, Göttingen, Germany
[4]Center of Anatomy, Georg-August-University, Göttingen, Germany
[5]Clinic of Orthopedic Surgery, Otto-von-Guericke-University, Magdeburg, Germany
[6]Institute for Applied Physics, Ludwig-Maximilians-University, Munich, Germany
[7]Research Laboratory, Clinic of Orthopedic Surgery, Tuebingen, Germany

Corresponding author: Thomas Pap (e-mail: thomas.pap@medizin.uni-magdeburg.de)

Abstract

To date, mesenchymal cells have only been associated with bone resorption indirectly, and it has been hypothesized that the degradation of bone is associated exclusively with specific functions of osteoclasts. Here we show, in aseptic prosthesis loosening, that aggressive fibroblasts at the bone surface actively contribute to bone resorption and that this is independent of osteoclasts. In two separate models (a severe combined immunodeficient mouse coimplantation model and a dentin pit formation assay), these cells produce signs of bone resorption that are similar to those in early osteoclastic resorption. In an animal model of aseptic prosthesis loosening (i.e. intracranially self-stimulated rats), it is shown that these fibroblasts acquire their ability to degrade bone early on in their differentiation. Upon stimulation, such fibroblasts readily release acidic components that lower the pH of their pericellular milieu. Through the use of specific inhibitors, pericellular acidification is shown to involve the action of vacuolar type ATPases. Although fibroblasts, as mesenchymal derived cells, are thought to be incapable of resorbing bone, the present study provides the first evidence to challenge this widely held belief. It is demonstrated that fibroblast-like cells, under pathological conditions, may not only enhance but also actively contribute to bone resorption. These cells should therefore be considered novel therapeutic targets in the treatment of bone destructive disorders.

Keywords: aseptic prosthesis loosening, bone resorption, dentin, fibroblasts, severe combined immunodeficient mouse

Introduction

Bone resorption by hyperplastic fibrous tissue is a characteristic feature of various disorders, and accumulating evidence suggests that transformed appearing, activated fibroblast-like cells play a key role in the pathogenesis of these conditions. One striking example is rheumatoid arthritis (RA), in which fibroblast-like synoviocytes constitute a considerable proportion of the hyperplastic synovium and are involved critically in the destruction of articular cartilage and bone [1]. Aseptic prosthesis loosening (APL), although apparently different at first sight, is also among these conditions and is characterized by the development of a synovial-like interface membrane (SLIM) between the prosthesis and the adjacent bone. Several studies have demonstrated similarities between the SLIM and the hyperplastic synovium in RA [2] and, intriguingly, there are a number of common features between fibroblast-like cells in RA and prosthesis loosening fibroblasts (PLFs) found at sites of bone resorption in APL. Recent data indicate that PLFs share some characteristic features of RA synovial fibroblasts, including anchorage-independent proliferation [3,4], escape of contact inhibition [5],

---

activation of tumour-associated pathways including proto-oncogenes [3] and alterations in apoptosis [6]. Apart form its relevance to orthopaedic surgery, APL is of general importance to our understanding of molecular mechanisms of fibroblast biology. Unlike the hyperplastic synovium in RA, which in the course of disease develops from a thin synovial membrane, the SLIM arises directly from progenitor cells in the bone marrow. Thus, PLFs probably originate directly from mesenchymal stem cells in the bone marrow and thereby render APL an interesting model for the differentiation of aggressive fibroblast-like cells at a bone surface.

Although it is well understood that, during the course of RA and APL, synovium and synovial-like membrane mediate the progressive destruction of bone, fibroblast-like cells have been implicated into this process only indirectly. Both RA synovial fibroblasts and PLFs release relevant matrix-degrading enzymes such as cathepsins, matrix metalloproteinases and membrane-type matrix metalloproteinases [7,8], and have been shown to secrete a number of factors that stimulate osteoclastic bone resorption [9,10]. In addition, recent data have demonstrated that fibroblast-like cells mediate the differentiation of macrophages into osteoclast-like cells [11,12]. The possibility that fibroblasts as mesenchymal-derived cells may resorb bone directly, however, has been rejected by some investigators [13]. Rather, it has been hypothesized that bone resorption is associated exclusively with specific functions of osteoclast-like cells that differentiate from the monocyte/macrophage lineage.

Here we demonstrate, for the first time, that fibroblast-like cells that develop at the bone surface in APL are capable of resorbing bone without the help of osteoclasts. In the severe combined immunodeficient (SCID) mouse coimplantation model, isolated human PLFs from late-stage APL produced signs of bone resorption. When examined by scanning electron microscopy, human PLFs that were cultured over extended periods of time on dentin slices exhibited morphological signs of bone resorption. Using PLFs from the developing periprosthetic tissue around knee prostheses of young, intracranially self-stimulated (ICSS) Wistar rats, we demonstrate that fibroblast-like cells acquire this ability early in the process of SLIM formation. We suggest that specific conditions, such as those found at the implant–prosthesis interface of joint arthroplasties, may induce the differentiation of fibroblast-like cells that have the potential to resorb bone independently of osteoclasts.

Material and method
Isolation of fibroblast-like cells
Tissue samples around loose joint arthroplasties were obtained from five patients undergoing revision surgery. Specimens were minced and digested enzymatically

(Dispase I, overnight). Released cells were grown in Dulbecco's modified Eagle's medium (Biochrom KG, Berlin, Germany) with 10% foetal calf serum (FCS; Gemini Biological Products, Calabasas, CA, USA) in a humidified 5% carbon dioxide atmosphere. After allowing the cells to adhere overnight, nonadherent cells were removed and the adherent cells were grown further over four passages.

Rat PLFs were obtained from the ICSS Wistar rat model (see below) accordingly. Following explanation of the rat prostheses together with the periprosthetic tissue, the SLIM tissue was removed [14], minced and digested enzymatically as was done with the human samples. Again, cells were grown in Dulbecco's modified Eagle's medium with 10% FCS, and there were no differences in terms of culturing the cells between rat and human PLFs.

Generation of human osteoclasts
Human peripheral blood was drawn from healthy adult donors. The blood was diluted 1:3 in Hank's balanced salt solution (BioxWhittaker Europe, Walkerville, MD, USA), layered on Biocoll Separation Solution (Biochrom KG) and centrifuged at $800\,g$ for 20 min. Isolated mononuclear cells were then washed three times in phosphate-buffered saline and cultured in minimal essential medium-α medium (GibcoBRL, Eggenstein, Germany) supplemented with 10% heat-inactivated FCS and 2% penicillin/streptomycin (GibcoBRL). Cells were plated in 24-well plates at 2×10^6 cells/ml in the presence of RANKL (receptor activator of nuclear factor-κB ligand) at $50\,ng/ml$ (Serotec, Düsseldorf, Germany) and macrophage colony-stimulating factor at $25\,ng/ml$ (R&D Systems, Wiesbaden, Germany) [15]. The cultures were fed by replacing half of the medium every 3 days. After 14 days, osteoclasts were incubated in phosphate-buffered saline containing 0.001% Pronase E and 0.02% EDTA for 5 min at room temperature. This incubation phase resulted in the detachment of cells other than osteoclasts from the dishes, arriving at a large number of highly enriched osteoclasts [16].

Characterization of fibroblast-like cells by flow cytometry
PLFs were trypsinized and fixed in 4% phosphate-buffered paraformaldehyde containing 1% FCS. For extracellular staining of human PLFs, the fibroblast surface specific antibody (clone AS02; Dianova, Hamburg, Germany) was used together with an anti-CD68 antibody (clone KP1; Signet laboratories Inc, Dedham, MA, USA). Cells were incubated with the primary antibodies for 30 min and a FITC-labeled goat anti-mouse IgG for 20 min. The analysis was performed on a FACS Calibur (Becton Dickinson, Heidelberg, Germany). For intracellular fluorescent-activated cell sorter (FACS) staining of rat PLFs, cells were treated with permeabilization buffer (0.1% saponine in phosphate-buffered saline, 0.1 M hepes and 1% FCS) and stained with anti-prolyl-4-hydroxylase (clone 6-9H6; DPC

Biermann, Bad Nauheim, Germany) and anti-CD68 (clone ED1; DPC Biermann), accordingly. All incubation and washing steps were performed in permeabilization buffer.

SCID mouse coimplantation experiments
Two-week-old, female SCID mice were provided by the Charles River GmbH (Sulzfeld, Germany). Normal human articular bone was obtained from the femoral head of a patient undergoing joint replacement surgery for severe trauma. The implantation of PLFs from three patients, together with bone, was performed as described for RA synovial fibroblasts [17]. After 60 days, mice were sacrificed and the implants removed. Tissue preparation included fixation in 4% buffered formalin and paraffin embedding according to standard procedures. Sections (4 μm) sections were stained with haematoxylin and eosin prior to examination.

Identification of human fibroblasts in the SCID mouse
Human fibroblasts were identified in the mouse sections with monoclonal mouse anti-vimentin antibodies (clone V9; Dako, Hamburg, Germany) using the Vector Mouse on Mouse immunodetection kit (Vector Laboratories Inc., Burlingame, CA, USA). Colour development was performed with 3,3′-diaminobenzidine, and sections were counterstained with methyl green. For negative control, mouse IgG was used instead of the primary antibodies.

Release of acidic components
The release of acidic components by human PLFs was measured using a cytosensor microphysiometer (Molecular Devices Corporation, Sunnyvale, CA, USA), as described previously [18]. Cells were activated by the cyclic influx of medium containing human recombinant tumour necrosis factor (TNF)-α (at a concentration between 1 and 300 ng/ml; Roche Biochemicals, Basel, Switzerland), ionomycin (10 μg/ml; Biomol, Hamburg, Germany) or control medium. For the ionomycin measurements, dimethylsulfoxide was included in the control medium. After 90 s, influx was stopped and acidification was determined over a period of 30 s [18,19]. Induction–response cycles were recorded for 45 min. To analyze the effects of ATPase inhibitors on the release of H^+, cells were treated by cyclic influx of medium containing amiloride (Calbiochem, Bad Soden, Germany) at concentrations between 100 μmol/l and 1 mmol/l, bafilomycin A_1 (Calbiochem) at concentrations between 10^{-8} mol/l and 10^{-5} mol/l, or control medium.

ICSS Wistar rat model of aseptic prosthesis loosening
Male Wistar rats (age 18–22 weeks, weight 350–400 g) were obtained from Harlan Winkelmann GmbH (Borchen, Germany). Implantation of the electrodes and the hemi-arthroplasties were performed under general anaesthesia, as previously described [14]. Briefly, bipolar electrodes were implanted into the medial forebrain bundle of the animals using a stereostactic device. For running exercise, the electrodes were connected to the running wheel (diameter 25 cm; width 8 cm) through an electronic switch. While running in the wheel, the rats received switch-triggered stimuli of 100 ms duration (current 0.3–1.0 mA). This electrical stimulation causes positive reinforcement in the rats and results in a running load that exceeds normal running activity by up to 100-fold [20]. For the implantation of prostheses, an arthrotomy of the left knee was performed through medial parapatellar incision. The anterior cruciate ligaments and the menisci were excised, whereas the posterior cruciate ligaments were preserved. The articular cartilage and the most superficial bone were resected from the tibial condyles and a hole was drilled into the tibial plateau. The prostheses were then inserted together with cement and compressed axially. Prostheses were examined by radiography. After implantation of the prostheses, rats were given 1 week to recover from surgery before the running exercise was initiated, as described above [20]. After 12 weeks, the knee joints containing the hemi-arthroplasties were harvested and used for isolation of fibroblast-like cells as well as histological examination following embedding in paraffin.

Dentin pit resorption assay
The dentine resorption assay was performed as described previously [21]. Dentin slices (300–400 μm thick; kindly provided by R Willmann, Institute of Zoology and Anthropology, University of Göttingen, Göttingen, Germany) were cut using a low-speed, diamond saw and cleaned by sonication in distilled water for 30 min. Fibroblasts from two APL patients or osteoclasts were seeded on the dentine slices and cultured in Dulbecco's modified Eagle's medium with 10% FCS and 2% penicillin/streptomycin, with and without TNF-α (10 ng/ml and 100 ng/ml). Dentin slices were fixed in 2.25% glutaraldehyde in 0.1 mol/l cacodylate buffer for 4 hours. They were then dehydrated in ethanol, dried in a critical point dryer, mounted on stubs, and Au:Pl coated in a cold spatter coater. The samples were examined by scanning electron microscopy (DSM 960; Zeiss, Jena, Germany).

Results
Human prosthesis loosening fibroblasts produce signs of bone resorption in the SCID mouse coimplantation model
To investigate the potential of human PLFs to resorb bone *in vivo*, fourth passage PLFs were coimplanted together with normal human bone into SCID mice. When investigated 60 days after implantation, PLFs were found to be attached to the bone surface (Fig. 1a), and the signs of bone resorption were most prominent at those sites at which the inert sponge used for the implantation of PLF had conferred close contact of the bone matrix with the inserted cells (Fig. 1b). This behaviour was seen with all PLFs. To demonstrate the human origin of the fibroblast-

Figure 1
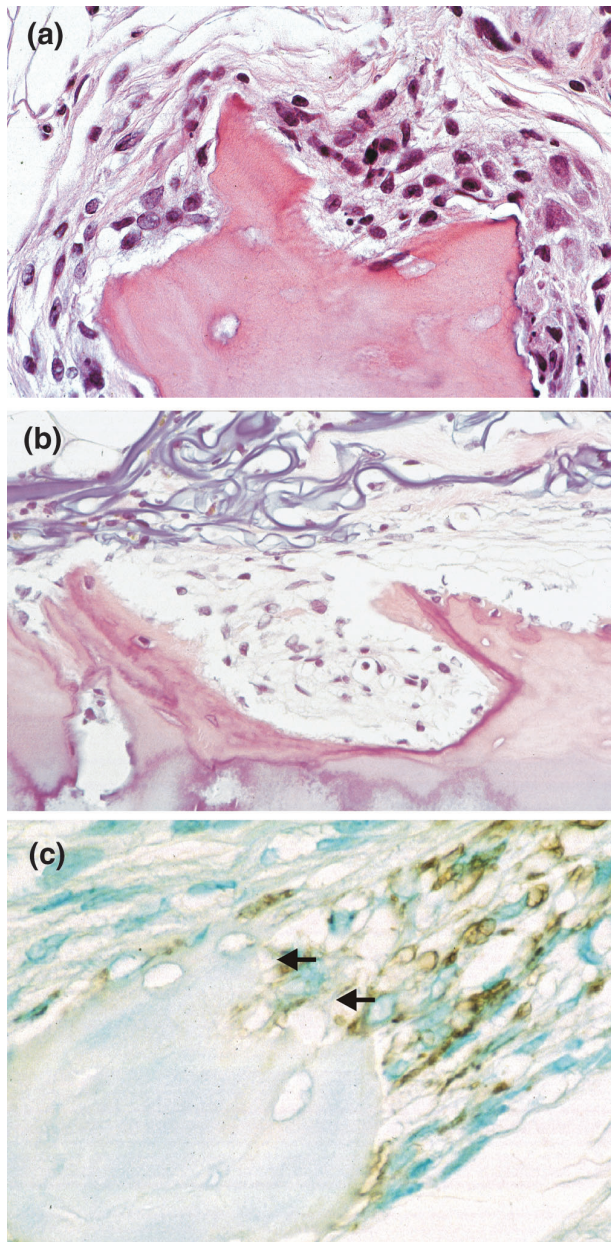
Fibroblast-like cells from the synovial-like interface membrane around loose joint arthroplasties produce signs of bone resorption in the severe combined immunodeficient (SCID) mouse model of matrix degradation. **(a)** and **(b)** Cultured prosthesis loosening fibroblasts (PLFs) were soaked into a collagen sponge and coimplanted with normal human bone under the renal capsule of SCID mice. After 60 days, histological evaluation showed attachment and invasion of the PLFs into the bone matrix (a, magnification ×630). The presence of PLFs in resorption pits was seen predominantly at sites at which the cells left the collagen sponge (b, magnification ×630). **(c)** Immunohistochemistry with antivimentin antibodies that recognize human fibroblasts but not mouse cells demonstrated the human origin of the fibroblast-like cells invading the bone. Human, vimentin-positive PLFs (arrowheads) were found abundantly in the SCID mouse sections (c, magnification ×630) and exhibited close contact to the resorption lacunae.

like cells that were found at sites of bone resorption in the SCID mice, we performed immunohistochemistry with monoclonal antibodies against the intermediate filament protein vimentin. These antibodies stain human cells of mesenchymal origin but specifically do not detect vimentin in mouse cells [22]. Immunohistochemistry showed the presence of vimentin-positive human PLFs in all samples, with most prominent staining at sites of bone resorption (Fig. 1c). Adjacent mouse tissue did not stain with the antibodies, and we observed no staining in isotype control experiments. To investigate potential effects of osteo-clasts, serial sections of all implants were screened for the presence of murine multinucleated, osteoclast-like cells. Interestingly, no such cells were seen at sites of bone resorption in any sample.

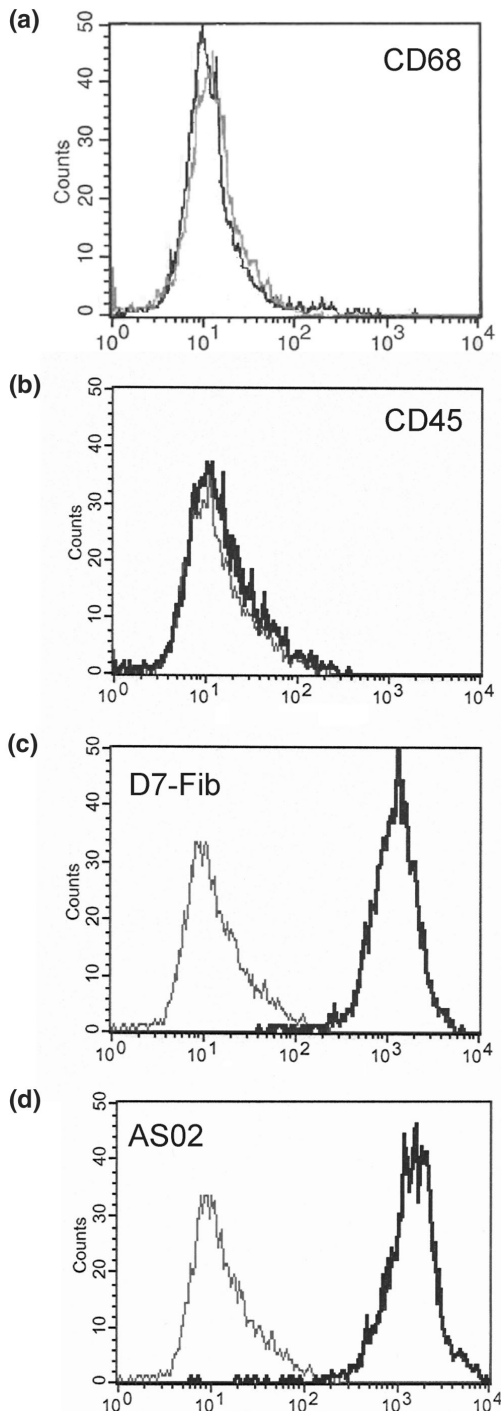**Characterization of human prosthesis loosening fibroblasts**

In order to exclude the contamination of PLF with cells of the monocyte/macrophage lineage, fourth passage PLFs were characterized by flow cytometry. FACS staining for the macrophage lineage marker CD68 was negative in all cultures (<0.1%; Fig. 2a). Moreover, the common leuco-cyte marker CD45 was also absent all cultures (<0.1%; Fig. 2b). In contrast, all of the PLFs (>99%) stained posi-tive for the fibroblast markers D7-Fib (Fig. 2c) and AS02 (Fig. 2d), confirming their identity as fibroblasts.

Resorption of dentin by human prosthesis loosening fibroblasts *in vitro*

Next, we investigated the ability of human PLFs to form resorption pits on whale dentin slices *in vitro*. PLFs were cultured on dentin slices for up to 4 weeks. Following removal of the cells, the formation of resorption pits was assessed by scanning electron microscopy. Pit formation by PLFs was compared with that by osteoclasts differenti-ated freshly *in vitro*. After 4 weeks on dentin slices, all PLFs exhibited morphological signs of bone resorption and produced characteristic resorption pits (Fig. 3a). Addi-tion of 10 ng/ml TNF-α (Fig. 3b) and 100 ng/ml TNF-α (Fig. 3c) enhanced the resorption of dentin by PLFs. Dentin slices on which no PLFs were cultured had a clear and smooth surface (Fig. 3d) and no superficial erosions were seen, thus excluding the possibility that the PLF-gen-erated resorption pits were artifacts. As expected, osteo-clasts produced characteristic resorption pits after 4–14 days (Fig. 3e) but there were considerable differ-ences between different osteoclast cultures (Fig. 3f).

Resorption of dentin by early differentiation prosthesis loosening fibroblasts from the ICSS Wistar rat model

In a further step, we sought to resolve the issue of whether bone resorption by fibroblast-like cells can be observed in fibroblasts from very early stages of SLIM formation. We implanted cemented tibial hemi-arthroplasties into the left knees of adult male Wistar rats and exposed the animals

Figure 2

(a) CD68

(b) CD45

(c) D7-Fib

(d) AS02

Characterization of cultured human prosthesis loosening fibroblasts (PLFs) by flow cytometry. **(a)** When compared with isotype control staining, analysis for the macrophage lineage marker CD68 showed no surface expression (<0.1%). **(b)** Also, no expression of the leucocyte common antigen CD45 was found on the cells (<0.1%). **(c)** However, fluorescent-activated cell sorter analysis with the fibroblast marker D7-Fib revealed the fibroblast nature of the cells (>99%). **(d)** In addition, more than 99% of the PLFs stained positive for the fibroblast marker AS02.

to excessive running in a running wheel by ICSS. As demonstrated previously [14], through positive reinforcement, ICSS results in running exercise that exceeds normal running activity of Wistar rats by up to 100-fold and leads to the development of a SLIM. This tissue shows all characteristics of the human SLIM but reflects very early stages of membrane formation. In the present study, we observed radiological signs of prosthesis loosening in the ICSS rats after 12 weeks (Fig. 4a). At this time point, we removed the periprosthetic SLIM, and histological examination revealed the presence of fibrous tissue with numerous fibroblast-like cells at sites of bone resorption (Fig. 4b), which corresponds to our previous observations. We used one part of the tissue to establish rat PLFs that were cultured over four passages. As done with the human PLFs, we excluded contamination of PLFs with cells of the monocyte/macrophage lineage by flow cytometry. There was no FACS staining with antibodies against CD68 (Fig. 4c) and more than 99% of rat PLFs stained positive with specific antibodies against prolyl-4-hydroxylase (Fig. 4d). Culturing the rat PLF on whale dentine slices for 4 weeks produced clear resorption pits (Fig. 4e, f).

Prosthesis loosening fibroblasts release acidic components upon stimulation

Because decalcification of the osseous matrix is a prerequisite for bone resorption, we investigated further the capability of the aggressive PLFs to release acidic components. We used ultrasensitive pH measurement in the direct pericellular environment with a cytosensor microphysiometer. Stimulation of PLFs with a calcium-dependent signal, as induced by the addition of ionomycin, resulted in rapid and significant release of acidic components. This was seen from an increase in the rate of acidification (maximum acidification r_{max} per cycle adjusted to the equilibrium value r_{eq}) by 5% after 20 min (data not shown). Stimulation of PLFs with TNF-α at different concentrations had even stronger effects. Although incubation of PLF with 1 ng/ml human recombinant TNF-α did not result in a significant stimulation of PLFs, adding 10, 100 and 300 ng/ml TNF-α resulted in substantial acidification of the pericellular milieu (Fig. 5a). Consistent with our data on TNF-α-mediated increase in the resorption of dentin, we detected maximal pericellular acidification (r_{max}/r_{eq}) of 15% with 10 ng/ml TNF-α after 20 min (Fig. 5b).

To test potential mechanisms through which PLFs release acidic components, we studied the effects of ATPase inhibitors on the pericellular acidification. Specifically, we used the ATPase inhibitor amiloride at high concentrations to inhibit vacuolar type (v-)ATPases [23], as well as the specific v-ATPase inhibitor bafilomycin A$_1$ [24], at different concentrations to analyze the time course of H$^+$ release as compared with that from untreated PLFs. Addition of amiloride at concentrations between 50 µmol/l and

Figure 3
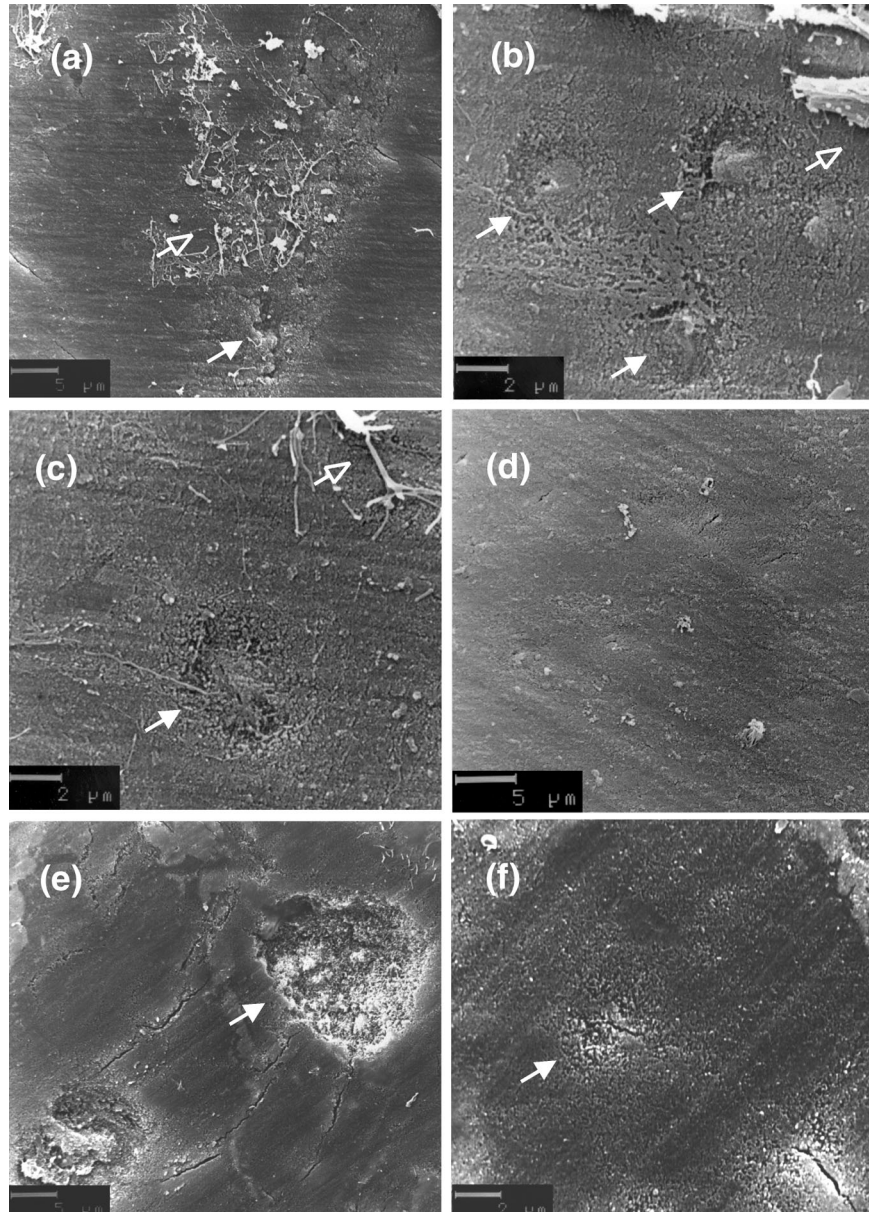
Human prosthesis loosening fibroblasts (PLFs) form resorption pits on whale dentin slices *in vitro*. **(a)** After 4 weeks of culture on dentin slices, PLFs exhibited morphological signs of bone resorption and produced characteristic signs of resorption pits (closed arrowheads) with remains of PLFs still *in situ* (open arrowheads). **(b)** Addition of 10 ng/ml tumour necrosis factor (TNF)-α to the medium enhanced the resorption of dentin, as seen from multiple resorption pits (closed arrowheads). Again, remains of PLFs were seen on the dentin slices (open arrowheads). **(c)** TNF-α 100 ng/ml also enhanced the size of the resorption pits generated by the PLFs (closed arrowhead). **(d)** Dentin slices on which no PLFs were cultured showed a clear and smooth surface and no erosions were seen. **(e)** Freshly differentiated osteoclasts that were used as controls produced characteristic resorption pits after 4 days (closed arrowhead). **(f)** However, there were considerable differences between osteoclast cultures, with some osteoclasts starting bone resorption only after 2 weeks (closed arrowhead).

1 mmol/l decreased the pericellular acidification by 32%, as seen from a significant increase in pericellular pH (Fig. 5c). Interestingly, this inhibition of H⁺ release was already evident after 3 min and remained stable for the total measuring time of 45 min without further increase. Pericellular pH returned to the initial values shortly after amiloride perfusion was stopped (Fig. 5d). Addition of the specific v-ATPase inhibitor bafilomycin A$_1$ resulted in up to 11% inhibition of pericellular acidification at concentrations of 10^{-6} mol/l (Fig. 5c). Again, the effect was seen at 5 min after addition of bafilomycin A$_1$, but in contrast to amiloride the secretion of protons was blocked irreversibly (Fig. 5e).

Figure 4
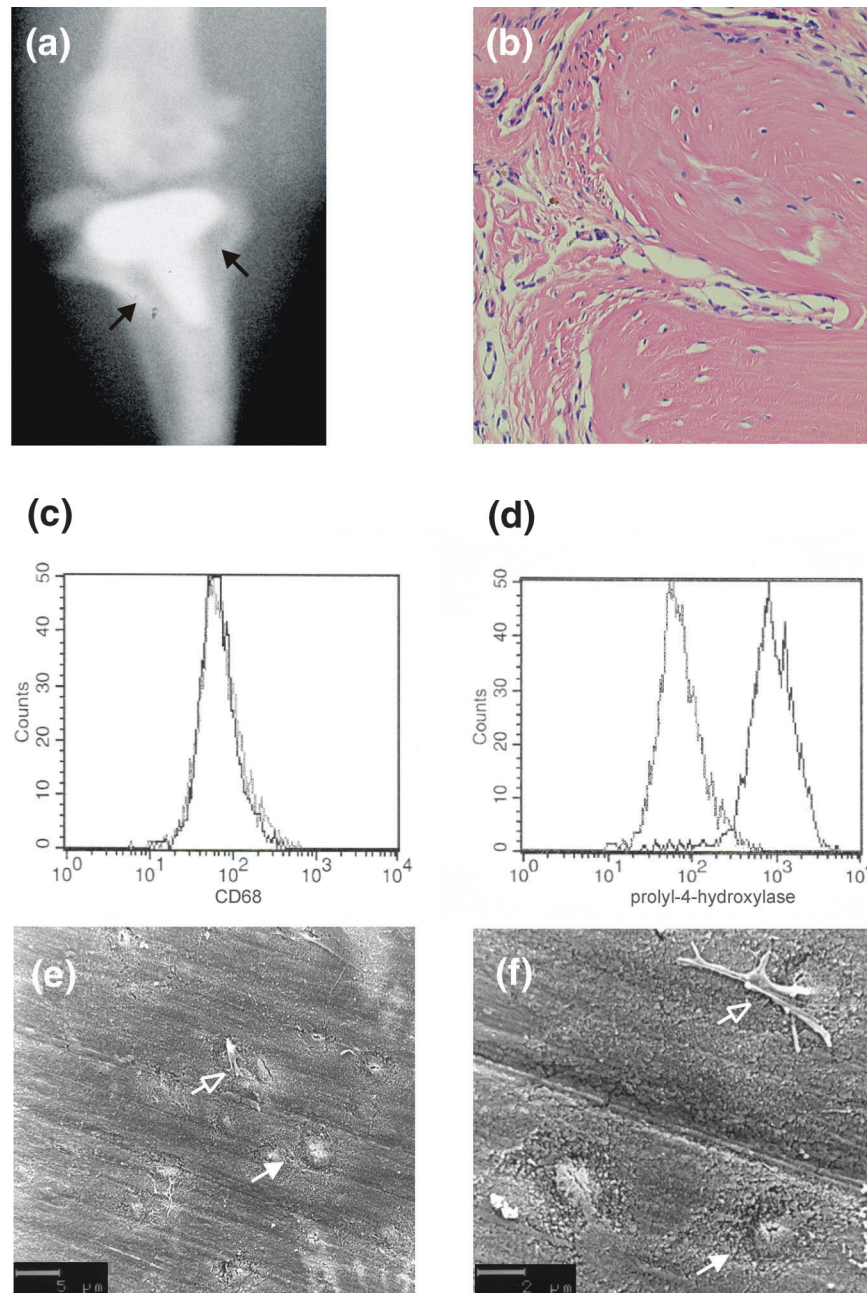
Resorption of whale dentin by early differentiation prosthesis loosening fibroblasts (PLFs) from the intracranially self-stimulated (ICSS) Wistar rat model of aseptic prosthesis loosening. **(a)** Cemented tibial hemi-arthroplasties that were implanted into the left knees of ICSS Wistar rats exhibited radiological signs of loosening (arrows) after 12 weeks of stimulated running in a running wheel. **(b)** Histological evaluation of the interface tissue between the prosthesis and the bone revealed the presence of loose fibrous tissue with fibroblast-like cells, particularly at sites of bone resorption. **(c)** and **(d)** Characterization of rat PLFs that were established from this tissue demonstrated the identity of these cells as fibroblasts in that more than 99% of the cells stained positive with specific antibodies against prolyl-4-hydroxylase (d), whereas there was no staining with antibodies against the monocyte lineage marker CD68 (c). **(e)** and **(f)** Culturing the rat PLFs on sperm whale dentine slices for 4 weeks produced clear resorption pits (closed arrowheads). Remains of the PLFs were seen in close contact with the resorption pits (open arrowheads).

Discussion

A growing body of evidence suggests a critical involvement of fibroblast-like cells in the destruction of extracellular matrix in various pathologies. Both APL and RA are charac-terized by aggressive growth and fibrotic remodelling. However, the mechanisms that result in the occurrence of aggressive fibroblast-like cells remain unclear. Specifically, it is not known in detail how environmental factors translate

Figure 5
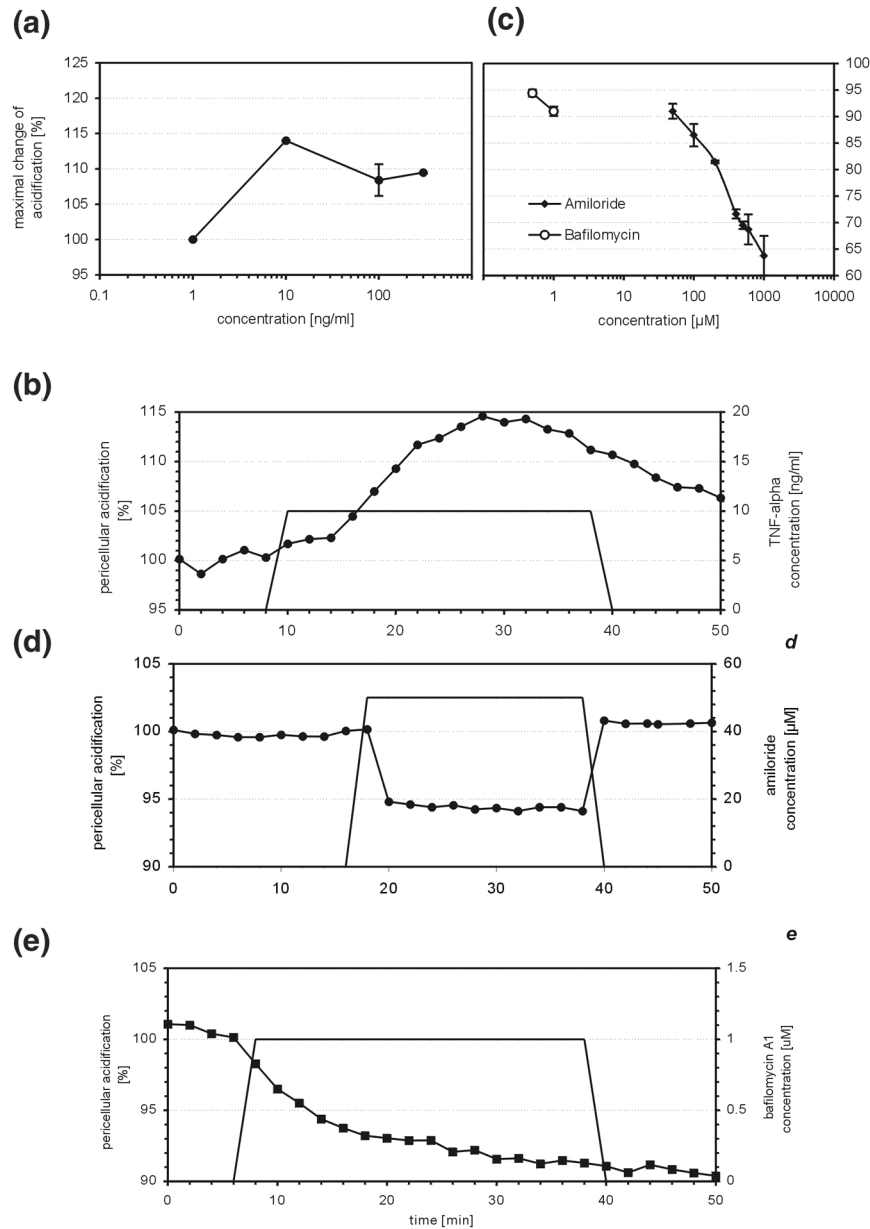
Release of acidic components by prosthesis loosening fibroblasts (PLFs). **(a)** and **(b)** Cytosensor measurement of PLFs stimulated with tumour necrosis factor (TNF)-α (1, 10 and 300 ng/ml) revealed the release of acidic components, with maximal pericellular acidification (r_{max}/r_{eq}) of 15% at 10 ng/ml TNF-α (A). Recording of the time curve with 10 ng/ml TNF-α showed a maximal acidification 15 min after the influx of TNF-α was started (b). **(c)** Incubation of PLFs with the ATPase inhibitors amiloride and bafilomycin A$_1$ at different concentrations decreased the pericellular acidification by a maximum of 32% with amiloride and 11% with bafilomycin A$_1$. **(d)** Inhibition of H$^+$ release by amiloride was recorded after 3 min and remained stable for the total measuring time of 45 min. Pericellular pH returned to the initial values shortly after the perfusion of amiloride was terminated. **(e)** The specific v-ATPase inhibitor bafilomycin A$_1$ at concentrations of 10^{-6} mol/l showed a clear effect shortly after its addition, and the H$^+$ secretion remained low even after discontinuation of bafilomycin A$_1$ infusion.

into the peculiar feautures that are exhibited by activated fibroblasts in different disorders. Based on the observation that in RA the aggressive phenotype of fibroblasts is maintained in the absence of inflammatory stimuli [17], it has

been hypothesized that activated fibroblasts are permanently imprinted or altered by the specific environment [25]. However, the question of how this imprinting is maintained in the cells remains to be answered.

In this context, APL is of special interest because it allows one to study directly the development of an aggressive synovial-like membrane from the bone marrow. To test the invasive behavior of PLFs, we used the well established SCID mouse model of matrix destruction [1,26]. In this model, fibroblast-like cells are implanted with normal human cartilage or bone into SCID mice. Because of their lack of a functional immune system, the mice do not reject the implants, and this allows study of the invasive behaviour of the fibroblasts in the absence of other human cells. The invasion of PLFs into cartilage in the SCID mouse model indicates that activated fibroblast-like cells, comparable to those in RA, can also be found in APL. At the same time, this observation raises the question as to whether differentiation of activated PLFs at the bone surface results in the ability of the cells to resorb osseous matrix, which is the target tissue in APL.

In the present study, we showed that fibroblasts that develop at the interface of bone and prosthesis in APL produce signs of bone resorption even in the absence of osteoclasts. Following coimplantation with femoral bone into SCID mice, resorption lacunae were found at sites of close contact between PLFs and the bone. The conclusion that osteoclasts may not be required for this process is derived from our experimental approach in the SCID mouse studies, in which we excluded the presence of human macrophages and osteoclasts in the implants. Immunohistological analysis clearly demonstrated the human nature of cells invading the coimplanted bone, and no murine osteoclasts were observed at sites of bone resorption in the SCID mice. However, we cannot completely exclude the possibility that murine osteoclasts that may be stimulated by the PLFs were present and contributed to our findings, and it may be argued that the complexity of this *in vivo* system makes it difficult to investigate all potential cellular interactions that may influence bone resorption. Therefore, it is of importance that isolated PLF produced clear resorption pits when cultured on dentin for 4 weeks and that dentin pit formation was enhanced visibly by addition of TNF-α. As compared with osteoclasts, PLF-generated resorption pits were similar to the early phases of osteoclastic bone resorption.

Of note, human PLFs in the present study were derived from late stage APL. Such tissue is rather fibrous [2] and, similar to the hyperplastic synovial membrane in RA, contains different subpopulations of fibroblast-like cells. Therefore, we investigated whether an ability of PLFs to resorb bone can also be identified in fibroblast-like cells from very early stages of SLIM formation. We used the ICSS Wistar rat model of APL [14] and demonstrated that the first signs of APL are found after 12 weeks. PLFs from the loose fibrous tissue that constitutes the SLIM at this early phase were capable of bone resorption, as shown on dentin slices. The morphology of these resorption pits was close to that seen in early osteoclasts, but the experimental approach and flow cytometry excluded contamination of the PLF cultures with cells of the monocyte/macrophage lineage. These findings complement our data from human cells by showing that PLFs acquire the ability to resorb bone early in their differentiation. Because the development of SLIM tissue cannot be studied in humans, our data demonstrate also value of the ICSS Wistar rat model for investigating early events in SLIM formation [14]. Moreover, this model provides a unique opportunity to study the characteristic features of fibroblasts during their development into aggressive matrix-degrading cells under standardized conditions.

Because it is known that fibroblasts both in RA and APL express enzymes, including membrane-type 1 matrix metalloproteinases and cathepsin K, which have been implicated in bone resorption [7,27], a major question related to the ability of fibroblasts to resorb bone is that of how these cells decalcify the osseous matrix before its degradation. Here, we show that, upon stimulation, PLFs release acidic components that result in a significant drop in the pericellular pH. This may be achieved by artificial calcium-dependent signaling with ionomycin and with TNF-α, that has been shown to be expressed abundantly within the SLIM [28]. Certainly, the drop in pH upon stimulation reflects metabolic activation of the PLFs, but it may also provide a mechanism by which these cells contribute to the decalcification of the bone matrix. Although the data presented here do not clarify specific mechanisms by which PLFs produce the acidic components, the expression of a specific H^+-ATPase on their cell membrane may potentially provide an explanation. It has been suggested that ATPases contribute to the H^+ secretion not only by osteoclasts [29] but also by other cells, including tubular kidney cells and cells of the inner ear [30]. It may therefore be hypothesized that similar mechanisms operate in PLFs. This notion is supported by the demonstration that specific inhibition of ATPases decreased pericellular acidification. Particularly, the use of bafilomycin A_1 – a specific inhibitor of v-ATPases – resulted in an irreversible inhibition of pericellular acidification. The observation that the release of H^+ is inhibited even after 5 min argues in favour of rapid membrane-associated mechanisms rather than slow transport of acidic equivalents from inside the cells.

It must be emphasized that osteoclasts constitute the major cell type involved in the degradation of bone not only under physiological conditions but also in disease. In the present study this is reflected clearly both by the extent and time course of osteoclast-mediated resorption of dentin. Notably, the most aggressive osteoclast cultures produced resorption pits even after 4 days, and those that showed less activity exhibited the first signs of dentin resorption after 14 days. In contrast, dentin resorption by PLFs was seen only after 4 weeks. In this regard, our data

are novel because they indicate that activated fibroblast-like cells differentiating under specific conditions such as APL may contribute directly to bone resorption. The data therefore contribute to the controversial discussion of whether degradation of calcified bone is linked exclusively to osteoclasts. Although studies by Chambers and Horton [31] failed to demonstrate bone resorption by mononuclear phagocytes, other observations suggested that tumour-infiltrating macrophages [32], as well as poly-methylmethacrylate-induced inflammatory macrophages [33], are capable of bone resorption in a specific interaction with stromal cells. In this context, the present results shed new light both on non-osteoclast-mediated bone resorption and on the role of fibroblast-like cells therein. The generation of an acidic milieu and the expression of bone-degrading enzymes may provide the tools for activated PLFs to degrade actively the osseous matrix in APL. This notion not only stresses the role of activated fibroblast-like cells in this process but may also provide novel targets to inhibit bone resorption.

Conclusion
Although the possibility that mesenchymal cells may resorb bone has been rejected by some investigators, we demonstrate here for the first time that fibroblasts not only enhance but also actively contribute to bone resorption. In prosthesis loosening, PLFs appear to acquire this feature early in their differentiation. It is concluded that fibroblasts should be considered a target for preventing bone resorption in prosthesis loosening and potentially other conditions of bone loss.

Competing interests
None declared.

References
1. Pap T, Muller-Ladner U, Gay RE, Gay S: **Fibroblast biology. Role of synovial fibroblasts in the pathogenesis of rheumatoid arthritis.** *Arthritis Res* 2000, **2**:361-367.
2. Goldring SR, Schiller AL, Roelke M, Rourke CM, O'Neil DA, Harris WH: **The synovial-like membrane at the bone-cement interface in loose total hip replacements and its proposed role in bone lysis.** *J Bone Joint Surg [Am]* 1983, **65**:575-584.
3. Xue C, Takahashi M, Hasunuma T, Aono H, Yamamoto K, Yoshino S, T, Nishioka K: **Characterisation of fibroblast-like cells in pannus lesions of patients with rheumatoid arthritis sharing properties of fibroblasts and chondrocytes.** *Ann Rheum Dis* 1997, **56**:262-267.
4. Lafyatis R, Remmers EF, Roberts AB, Yocum DE, Sporn MB, Wilder RL: **Anchorage-independent growth of synoviocytes from arthritic and normal joints. Stimulation by exogenous platelet-derived growth factor and inhibition by transforming growth factor-beta and retinoids.** *J Clin Invest* 1989, **83**:1267-1276.
5. Grimbacher B, Aicher WK, Peter HH, Eibel H: **TNF-alpha induces the transcription factor Egr-1, pro-inflammatory cytokines and cell proliferation in human skin fibroblasts and synovial lining cells.** *Rheumatol Int* 1998, **17**:185-192.
6. Franz JK, Pap T, Hummel KM, Nawrath M, Aicher WK, Shigeyama Y, Muller-Ladner U, Gay RE, Gay S: **Expression of sentrin, a novel antiapoptotic molecule, at sites of synovial invasion in rheumatoid arthritis.** *Arthritis Rheum* 2000, **43**:599-607.
7. Pap T, Pap G, Hummel KM, Franz JK, Jeisy E, Sainsbury I, Gay RE, Billingham M, Neumann W, Gay S: **Membrane-type-1 matrix metalloproteinase is abundantly expressed in fibroblasts and osteoclasts at the bone-implant interface of aseptically loosened joint arthroplasties in situ.** *J Rheumatol* 1999, **26**:166-169.
8. Pap T, Shigeyama Y, Kuchen S, Fernihough JK, Simmen B, Gay RE, Billingham M, Gay S: **Differential expression pattern of membrane-type matrix metalloproteinases in rheumatoid arthritis.** *Arthritis Rheum* 2000, **43**:1226-1232.
9. Bertolini DR, Nedwin GE, Bringman TS, Smith DD, Mundy GR: **Stimulation of bone resorption and inhibition of bone formation in vitro by human tumour necrosis factors.** *Nature* 1986, **319**:516-518.
10. Xu JW, Konttinen YT, Waris V, Pätiälä H, Sorsa T, Santavirta S: **Macrophage-colony stimulating factor (M-CSF) is increased in the synovial-like membrane of the periprosthetic tissues in the aseptic loosening of total hip replacement (THR).** *Clin Rheumatol* 1997, **16**:243-248.
11. Takayanagi H, Oda H, Yamamoto S, Kawaguchi H, Tanaka S, Nishikawa T, Koshihara Y: **A new mechanism of bone destruction in rheumatoid arthritis: synovial fibroblasts induce osteoclastogenesis.** *Biochem Biophys Res Commun* 1997, **240**:279-286.
12. Sabokbar A, Fujikawa Y, Neale S, Murray DW, Athanasou NA: **Human arthroplasty derived macrophages differentiate into osteoclastic bone resorbing cells.** *Ann Rheum Dis* 1997, **56**:414-420.
13. Teitelbaum SL: **Bone resorption by osteoclasts.** *Science* 2000, **289**:1504-1508.
14. Pap G, Machner A, Rinnert T, Horler D, Gay RE, Schwarzberg H, Neumann W, Michel BA, Gay S, Pap T: **Development and characteristics of a synovial-like interface membrane around cemented tibial hemiarthroplasties in a novel rat model of aseptic prosthesis loosening.** *Arthritis Rheum* 2001, **44**:956-963.
15. Lader CS, Scopes J, Horton MA, Flanagan AM: **Generation of human osteoclasts in stromal cell-free and stromal cell-rich cultures: differences in osteoclast CD11c/CD18 integrin expression.** *Br J Haematol* 2001, **112**:430-437.
16. Tezuka K, Sato T, Kamioka H, Nijweide PJ, Tanaka K, Matsuo T, Ohta M, Kurihara N, Hakeda Y, Kumegawa M: **Identification of osteopontin in isolated rabbit osteoclasts.** *Biochem Biophys Res Commun* 1992, **186**:911-917.
17. Müller-Ladner U, Kriegsmann J, Franklin BN, Matsumoto S, Geiler T, Gay RE, Gay S: **Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafted into SCID mice.** *Am J Pathol* 1996, **149**:1607-1615.
18. Parak WJ, Dannohl S, George M, Schuler MK, Schaumburger J, Gaub HE, Muller O, Aicher WK: **Metabolic activation stimulates acid production in synovial fibroblasts.** *J Rheumatol* 2000, **27**:2312-2322.
19. Owicki JC, Bousse LJ, Hafeman DG, Kirk GL, Olson JD, Wada HG, Parce JW: **The light-addressable potentiometric sensor: principles and biological applications.** *Annu Rev Biophys Biomol Struct* 1994, **23**:87-113.
20. Pap G, Eberhardt R, Sturmer I, Machner A, Schwarzberg H, Roessner A, Neumann W: **Development of osteoarthritis in the knee joints of Wistar rats after strenuous running exercise in a running wheel by intracranial self-stimulation.** *Pathol Res Pract* 1998, **194**:41-47.
21. Boyde A, Ali NN, Jones SJ: **Resorption of dentine by isolated osteoclasts in vitro.** *Br Dent J* 1984, **156**:216-220.
22. Bohn W, Wiegers W, Beuttenmüller M, Traub P: **Species-specific recognition patterns of monoclonal antibodies directed against vimentin.** *Exp Cell Res* 1992, **201**:1-7.
23. McKinney LC, Moran A: **Regulation of intracellular pH in J774 murine macrophage cells: H^+ extrusion processes.** *Am J Physiol* 1995; **268**:C210-C217.

24. Chatterjee D, Chakraborty M, Leit M, Neff L, Jamsa-Kellokumpu S, Fuchs R, Baron R: **Sensitivity to vanadate and isoforms of subunits A and B distinguish the osteoclast proton pump from other vacuolar H$^+$ ATPases.** *Proc Natl Acad Sci USA* 1992, **89**: 6257-6261.

25. Han Z, Boyle DL, Chang L, Bennett B, Karin M, Yang L, Manning AM, Firestein GS: **c-Jun N-terminal kinase is required for metalloproteinase expression and joint destruction in inflammatory arthritis.** *J Clin Invest* 2001, **108**:73-81.

26. Pap T, Aupperle KR, Gay S, Firestein GS, Gay RE: **Invasiveness of synovial fibroblasts is regulated by p53 in the SCID mouse in vivo model of cartilage invasion.** *Arthritis Rheum* 2001, **44**: 676-681.

27. Hummel KM, Petrow PK, Franz JK, Müller-Ladner U, Aicher WK, Gay RE, Bromme D, Gay S: **Cysteine proteinase cathepsin K mRNA is expressed in synovium of patients with rheumatoid arthritis and is detected at sites of synovial bone destruction.** *J Rheumatol* 1998, **25**:1887-1894.

28. Xu JW, Konttinen YT, Lassus J, Natah S, Ceponis A, Solovieva S, Aspenberg P, Santavirta S: **Tumor necrosis factor-alpha (TNF-alpha) in loosening of total hip replacement (THR).** *Clin Exp Rheumatol* 1996, **14**:643-648.

29. Nakamura H, Moriyama Y, Futai M, Ozawa H: **Immunohistochemical localization of vacuolar H(+)-ATPase in osteoclasts of rat tibiae.** *Arch Histol Cytol* 1994, **57**:535-539.

30. Karet FE, Finberg KE, Nelson RD, Nayir A, Mocan H, Sanjad SA, Rodriguez-Soriano J, Santos F, Cremers CW, Di Pietro A, Hoffbrand BI, Winiarski J, Bakkaloglu A, Ozen S, Dusunsel R, Goodyer P, Hulton SA, Wu DK, Skvorak AB, Morton CC, Cunningham MJ, Jha V, Lifton RP: **Mutations in the gene encoding B1 subunit of H$^+$-ATPase cause renal tubular acidosis with sensorineural deafness.** *Nat Genet* 1999, **21**:84-90.

31. Chambers TJ, Horton MA: **Failure of cells of the mononuclear phagocyte series to resorb bone.** *Calcif Tissue Int* 1984, **36**: 556-558.

32. Quinn JM, Athanasou NA: **Tumour infiltrating macrophages are capable of bone resorption.** *J Cell Sci* 1992, **101**:681-686.

33. Quinn J, Joyner C, Triffitt JT, Athanasou NA: **Polymethylmethacrylate-induced inflammatory macrophages resorb bone.** *J Bone Joint Surg Br* 1992, **74**:652-658.

Correspondence

Thomas Pap, MD, Division of Experimental Rheumatology, University Hospital Magdeburg, Leipziger-Str. 44, D-39120 Magdeburg, Germany. Tel: +49 391 6713314; fax: +49 391 6715447; e-mail: thomas.pap@medizin.uni-magdeburg.de