Interface Tissue Fibroblasts from Loose Total Hip Replacement Prosthesis Produce Receptor Activator of Nuclear Factor-κB Ligand, Osteoprotegerin, and Cathepsin K

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ABSTRACT. ***Objective.*** The highly osteolytic interface tissue between the bone and loosening total hip prosthesis is characterized by low pH, formation of foreign body giant cells, osteoclasts, and production of receptor activator of nuclear factor-κB (RANKL) and cathepsin K. We hypothesized that fibroblasts in the interface tissue may form a source for RANKL production.

Methods. Primary interface tissue fibroblasts, fibrous joint capsule fibroblasts, and trabecular bone osteoblasts were stimulated with tumor necrosis factor-α (TNF-α), interleukin 1ß (IL-1ß), IL-6, IL-11, or 1α,25-$(OH)_2$ vitamin D_3. Cellular RANKL and released cathepsin K were detected by Western blotting. RANKL in cell lysates and osteoprotegerin (OPG) in cell culture medium were measured by ELISA. RANKL, OPG, and cathepsin K mRNA were measured with quantitative reverse transcriptase polymerase chain reaction.

Results. Interface tissue fibroblasts were found to produce RANKL. 1α,25-$(OH)_2$ vitamin D_3 stimulation increased RANKL mRNA expression. TNF-α was found to be the most potent OPG inducer in interface tissue fibroblasts. Cathepsin K mRNA production in fibroblasts was upregulated roughly 3-fold ($p < 0.01$) after 1α,25-$(OH)_2D_3$ stimulation, and both pro- and active cathepsin K protein was released to fibroblast culture media.

Conclusion. Interface tissue fibroblasts are able to produce RANKL, OPG, and cathepsin K and may contribute indirectly and directly to pathologic periprosthetic collagenolysis and bone destruction. (J Rheumatol 2005;32:713–20)

Key Indexing Terms:

CATHEPSIN K FIBROBLAST IMPLANTS
OSTEOPROTEGERIN RANKL VITAMIN D

Total hip replacement (THR) surgery is a cost-effective treatment for endstage hip disease of osteoarthritis and rheumatoid arthritis. A significant problem of THR surgery is longterm aseptic loosening of primarily well implanted prostheses. Wear debris derived from the gliding surfaces of the prosthesis lead to local accumulation and activation of macrophages that are considered the key mediators of inflammation associated with aseptic loosening[1-3]. Aseptic loosening always involves development of fibroblast-rich synovial membrane-like interface tissue that grows between the implant and bone[4,5]. As a consequence, loose prostheses must be replaced in an expensive and technically difficult revision operation.

Low pH and high cathepsin K production in the interface tissue lead to dissolution of hydroxyapatite from the periprosthetic bone bed, followed by cathepsin K-mediated bone collagen degradation[6]. Cathepsin K production and giant cell and osteoclast formation seem to be mediated by the receptor activator of nuclear factor-κB ligand (RANKL) system[7-9]. Early osteoclast precursors fuse and differentiate to osteoclasts when osteoblast-produced RANKL binds to their cell surface receptor, known as receptor activator of NF-κB (RANK). This is inhibited by a soluble decoy receptor, osteoprotegerin (OPG)[10-14].

Interleukin 1ß (IL-1ß), IL-11, tumor necrosis factor-α (TNF-α), dexamethasone, 1α,25-$(OH)_2$ vitamin D_3,

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Supported by the Academy of Finland, TEKES, Sigrid Jusélius Foundation, the Invalid Foundation, Helsinki University Hospital (TYH 0341, TYH 1233, TYH 2267), and Biomedicum Helsinki research funds. J. Mandelin was supported by the University of Helsinki Science Foundation and Emil Aaltonen Foundation (Young Investigator grants).

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Accepted for publication December 31, 2004.

parathyroid hormone, and prostaglandin E_2 have been shown to increase RANKL mRNA production in osteoblasts, whereas transforming growth factor-ß (TGF-ß) may suppress it. Some of the same cytokines also regulate OPG mRNA levels. Although reports differ, this can be summarized as follows: IL-1α and IL-1-ß, TNF-α and TNF-ß, bone morphogenetic protein-2, TGF-ß, 1α,25-$(OH)_2$ vitamin D_3, and 17ß-estradiol increase and parathyroid hormone and prostaglandin E_2 can suppress OPG mRNA production in osteoblasts[15-17].

Although the regulation of RANKL and OPG production has been studied in detail in osteoblasts and some studies have addressed the regulation of osteoclastogenesis by the RANKL system in rheumatoid synoviocytes[18], little is known about the regulation of these molecules in interface tissue fibroblasts. We investigated how the fibroblasts of interface tissue and hip joint capsule compare to osteoblasts isolated from femoral epiphysis in respect to RANKL, OPG, and cathepsin K production. This study provides quantitative analysis of RANKL, OPG, and cathepsin K expression in fibroblasts of both healthy and diseased origin, and evidence that fibroblasts have potential to contribute both directly and indirectly to peri-implant tissue destruction in aseptic loosening of total joint replacement prostheses.

MATERIALS AND METHODS

Patients and samples. Synovial membrane-like interface tissue samples (n = 8) were collected from areas adjacent to osteolytic lesions between the prostheses or cement and femoral cortical bone from patients undergoing revision operation due to aseptic loosening of THR. There were no clinical or laboratory signs of infection in any patient. The indication for primary THR was osteoarthritis in all cases. Four of the patients were men and 2 were women, with mean age 69.5 ± 11 years (mean ± SD).

Hip joint capsule tissue samples (n = 6) were obtained from patients undergoing primary THR for osteoarthritis of the hip. Dense collagenous capsular tissue was selected as a source of native connective tissue fibroblasts. Loose connective tissue was cut away from the samples before culture. Of these patients, 5 were women and one a man, their mean age being 58 ± 12 years.

Femoral heads from patients (n = 6) undergoing hemiarthroplasty due to a fracture of the femoral neck were used for osteoblast isolation. Five of the patients were women and one was a man. The mean age of these patients was 83 ± 11 years.

Immunocytochemistry. Cryostat sections (6 µm) were mounted on Dako capillary microscope slides (TechMate™, Dako, Glostrup, Denmark) and fixed in cold acetone for 5 min at 4°C, then washed in 0.01 M phosphate-buffered 150 mM saline (PBS, pH 7.4) for 5 min. The slides were immunostained with Dako TechMate™ Horizon automatic immunostainer at 22°C. The following protocol was used: (1) incubation with the primary antibody solution for 25 min. The concentrations of the antibodies were: rabbit anti-cathepsin K IgG 0.17 µg/ml (a kind gift from Dr. Eiko Sakai), mouse anti-human RANKL IgG_{2B} 1 µg/ml, and mouse anti-human OPG IgG_1/κ 1.25 µg/ml (both from R&D Systems, Minneapolis, MN, USA); (2) incubation with biotinylated secondary antibody for 25 min; (3) peroxidase blocking for 25 min; (4) incubation with peroxidase-conjugated streptavidin 3 times for 3 min; (5) incubation in horseradish peroxidase (HRP) substrate buffer; and finally (6) color development with 3,3'-diaminobenzidine tetrahydrochloride (ChemMate™ detection kit) for 5 min. Between each step, the sections were washed with Dako ChemMate™ washing buffers 3 times and dried with absorbent pads. After immunostaining, the sections were removed from the machine, counterstained with hematoxylin, washed, dehydrated in ethanol series, cleared in xylene, and mounted. Normal rabbit IgG or monoclonal mouse IgG_{2B}/κ or IgG_1/κ of irrelevant specificity was used with the same concentrations as and instead of the primary antibodies.

Cell culture. Soft tissue samples were minced into pieces with a sterile scalpel in a laminar flow hood. The explants were left overnight in RPMI-1640 medium (Biomedicum Helsinki, Helsinki, Finland) containing 10% fetal bovine serum (FBS; BioWhittaker, Liege, Belgium) with 1000 U/ml penicillin and 1 mg/ml streptomycin (10×) solution. The next day, the media were changed to basal RPMI with 10% FBS media and 100 U penicillin and 0.1 mg streptomycin (1× solution). The medium was changed twice a week for 3 weeks, and when roughly 80% monolayer confluence was reached the explants were removed, and the cells were subcultured 1:3 until confluent. Passages 2–4 were used for subsequent experiments.

Trabecular bone of the femoral heads was cut into pieces for osteoblast cultures. The pieces were washed several times with 10 mM phosphate buffered 150 mM saline, pH 7.4 (PBS) and subjected to collagenase (Collagenase XI; Sigma, St. Louis, MO, USA) treatment for 4 × 15 min. After washes with PBS, the explants were washed once with a culture medium (Dulbecco's modified Eagle's medium, DMEM) without D-glucose and sodium pyruvate, (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS and 10× penicillin and streptomycin, and cultured overnight. On the following day, the medium was changed to basal DMEM with 10% FBS and 1× antibiotics. Then the samples were processed as described above.

For experiments, the cells were grown to confluence in 6-well plates. The number of cells was about 2×10^5 cells/well. The cells were stimulated with TNF-α (0.05 ng/ml; R&D Systems), IL-1ß (0.01 ng/ml, R&D Systems), IL-6 (0.5 ng/ml, R&D Systems), IL-11 (0.1 ng/ml, R&D Systems), or 1α,25-$(OH)_2$ vitamin D_3 (1.0×10^{-8} mol/l; Calbiochem, San Diego, CA, USA) for 72 h, then culture media were collected. Each stimulation was made in triplicate. Cells were washed with PBS and lysed with 1% Triton X-100 in PBS. Samples were centrifuged to remove cell debris and cell homogenates were stored at –20°C until used. For RNA isolation, the cells were stimulated as above for 24 h, washed with PBS and lysed with TRIzol (Invitrogen).

Phenotype assessment of cultures. Phenotype assessment and purity of the cultures was determined by immunofluorescence with the following antibodies: fibroblast marker 5B5 (Dako), monocyte marker CD68 KP1 (Dako), endothelial cell marker CD31 (Dako), and smooth muscle myosin (BioGenex, San Ramon, CA, USA). Cells were cultured on glass coverslips, washed, fixed with 2% paraformaldehyde (Sigma) for 20 min at 22°C, treated with 0.2% TX-100 (Sigma) for 10 min at 22°C, and after washing, blocked with 3% goat normal serum, followed by incubation with the antibodies for 30 min at 22°C. After washing, the appropriate Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) secondary antibody was applied to the coverslips and incubated 30 min at 22°C. Finally, the coverslips were washed and stained with 4,6-diamidino-2-phenylindole (DAPI; Sigma) to visualize the nuclei, mounted with Glycergel (Dako) on microscope slides, and kept in the dark at 4°C before analysis. Osteoblast phenotype was confirmed with von Kossa staining.

Western blotting. For RANKL analysis, 5 µg cell lysate and, for cathepsin K analysis, 15 µg culture medium were boiled for 5 min in SDS gel loading buffer before electrophoresis. Recombinant soluble 28 kDa RANKL protein (200 ng/lane; Alexis Corp., Lausen, Switzerland) and human giant cell tumour of bone lysates (75 µg) were used for positive controls. Electrophoresis was performed in 10% polyacrylamide slab gel. After electrophoresis, the gels were blotted onto nitrocellulose membrane (Bio-Rad, Richmond, CA, USA). The membrane was blocked overnight using 3% bovine serum albumin (BSA; Sigma) in Tris buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 7.5) and washed in washing buffer (0.1% Tween 20, 50 mM Tris-HCl, 0.5 M NaCl, pH 7.5). After washes, the membranes were incubated with biotinylated rabbit anti-human sRANKL IgG

detection antibody (0.25 µg/ml; PeproTech, Rocky Hill, NJ, USA) or affinity purified goat anti-human cathepsin K IgG (0.4 µg/ml; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in washing buffer containing 2% BSA for 1 h. This was followed with washes and incubation for 1 h at 22°C with alkaline phosphatase conjugated ExtrAvidine (1:5000 in washing buffer containing 2% BSA; Sigma) or with alkaline phosphatase conjugated rabbit anti-goat IgG (1:5000 in washing buffer containing 2% BSA; Jackson Immunoresearch Laboratories, West Grove, PA, USA). The membranes were washed with washing buffer for 30 min with at least 3 changes of buffer followed by a final wash in TBS. The alkaline phosphatase-binding sites were revealed with color development solution (alkaline phosphatase conjugate substrate kit; Bio-Rad) containing a mixture of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium (NBT). The color reaction was stopped at 30 min by washing the membranes in distilled water for 10 min.

RANKL and OPG ELISA. RANKL ELISA was performed from cell lysates with sRANKL kit (Biomedica, Vienna, Austria). Samples were diluted 1:5 with PBS. Then 100 µl of diluted sample was added to 96-well microtiter plate wells and 100 µl of detection antibody was added. A 7 point standard diluted from 2 ng/ml to 31.25 pg/ml was prepared from kit stock standard in PBS. A base control provided by the kit was diluted 1:5 and used to control each individual assay. Duplicates were used for samples and standards. Plates were incubated 16–24 h at 4°C and washed 3 times with 350 µl washing buffer. After washes, 200 µl streptavidin-HRP conjugate was added to the wells, followed by incubation for 1 h at 22°C under continuous gentle shaking. The plates were washed 3 times, then 200 µl tetramethylbenzidine (TMB) color substrate solution was added to the wells and incubated in the dark for 20 min. The reaction was stopped with 50 µl stop solution and the absorbance was measured at 450 nm.

For OPG ELISA, Nunc MaxiSorp™ (Nunc, Rochester, NY, USA) plates were coated with monoclonal mouse anti-human OPG IgG_{2a} (2 µg/ml in PBS; R&D Systems, clone 69127.11) overnight at room temperature. The plates were washed 3 times with PBS containing 0.05% Tween 20, pH 7.4, and blocked with 1% BSA and 5% sucrose in PBS for 1 h. The blocking buffer was removed and 100 µl of media samples, diluted 1:100 in 1% BSA in PBS to adjust the sample to the dynamic range of the assay, were added to each well for 1.5 h at 22°C. Recombinant human OPG/Fc chimera (R&D Systems) at 2 ng/ml to 31.25 pg/ml was used as a standard. After washes the bound OPG was detected with biotinylated anti-human OPG IgG (100 ng/ml; R&D Systems) diluted in 1% BSA in PBS. The plates were washed and 1:70,000 diluted alkaline phosphatase conjugated ExtrAvidin was added. Unbound ExtrAvidin was washed away and the color was developed using Sigma Fast™ p-nitrophenyl phosphate tablet sets (pNPP substrate). The absorbance was measured at 405 nm.

Quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated by TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA was measured spectrophotometrically and its quality was confirmed with ethidium bromide-stained 1% agarose gels under UV light. RNA was DNase treated (Promega, Madison, WI, USA) and 2 µg was used to prepare primary cDNA using $(dT)_{12\text{-}18}$ primers and SuperScript enzyme, followed by RNase H treatment (Invitrogen). Quantitative PCR was run using 0.2 µg first-strand cDNA, 0.5 mM primers, and 0.2 mM TaqMan probes (Table 1) in LightCycler™ PCR mix in a LightCycler™ PCR machine (Roche Molecular Biochemicals, Mannheim, Germany). The identity of the product of one sample from each group and the positive control was verified by sequencing 50 ng isolated (QIAquick; Qiagen, Chatsworth, CA, USA) amplicon using an automated Applied Biosystems 373A sequencer. Probes had FAM reporter dye (6-carboxy-fluorescein) at the 5' end and TAMRA quencher dye (6-carboxy-tetramethyl-rhodamine) at the 3' end, which was also phosphorylated[19]. Serial 1:10 dilutions of human genomic DNA were used to determine the copy numbers of the amplicon in relation to porphobilinogen deaminase (PBGD) mRNA copies. The copy numbers of the housekeeping PBGD were similar in all samples. PBGD was used as a standard gene and as a marker of successful cDNA synthesis. All primers were designed so that they were located inside one exon to make it possible to use genomic DNA as a standard. The copy numbers of mRNA molecules were determined at least twice for all samples. The cDNA synthesis reaction was also performed without RT enzyme, followed by amplification of PBGD to exclude the possibility of genomic DNA contamination.

Statistical analysis. Repeated measures ANOVA with Bonferroni's multiple comparison test was used to compare different stimulations. One-way ANOVA with Bonferroni's multiple comparison test was used to compare different cell types. Spearman correlations were used to analyze the correlations between mRNA and protein levels. All tests were performed with GraphPad Prism, v. 3.02 for Windows (GraphPad Software, San Diego, CA, USA). All results are expressed as mean ± SEM.

RESULTS

Histology of synovial membrane-like interface tissue. The interface tissue samples from which the cell cultures were derived were characterized by a foreign body-type reaction with histiocytosis and fibrosis. A synovial lining-like structure, usually 1–3 cell layers thick, was found in all samples. The stroma consisted of the cell-rich areas containing macrophage-like cells and giant cells, and the fibrotic areas dense collagenous fibers and fibroblast-like cells (Figure 1A). Polyethylene wear debris was visible under polarized microscopy in each case. Metal particles were small and black. Bone cement particles usually appeared as irregular voids as they were dissolved during tissue processing. Thus, the samples used in our study were representative for aseptic loosening of total hip implants[4,5].

Immunohistochemistry of synovial membrane-like interface tissue. Intense and abundant cathepsin K immunoreactivity was detected in all interface tissue samples. Particle-associated foreign body giant cells and mononuclear cells contained cathepsin K. Cells in synovial lining-like structure and collagenous areas also disclosed cathepsin K immunoreactivity (Figure 1B). RANKL immunoreactivity was detected in all synovial membrane-like interface tissue

Table 1. Sequences of primers and probes used in quantitative PCR. The genes and their sequence accession numbers are shown. Primers were designed and tested so that they locate within one exon in all genes. Probes anneal to minus strand.

Gene	Acc. No.	5' Primer	3' Primer	Probe	Length, 6p
OPG	U94332	GGCATTCTTCAGGTTTGCTGTTCCTA	AGCTGTGTTGCCGTTTTATCCTCTCT	mATTTGCCTGGCACCAAAGTAAACGCAxp	125
RANKL	AF019047	CCAACATTTGCTTTCGACATCATGAA	TGACCAATACTTGGTGCTTCCTCCT	mACGTCACTAAAACCAGCATCAAAATCCCAAxp	143
Cathepsin K	X82153	CAGTGAAGAGGTGGTTCAGA	AGAGTCTGGGGCTCTACCTT	mTCCCGCAGTAATGACACCCTTxp	109
PBGD	M95623.1	GGGAAACCTCAACACCCGGCT	ACCCGGTTGTGCCAGCCCAT	mATCCTGGCAACAGCTGGCCTGCxp	105

m: 6-carboxy-fluorescein (FAM); x: 6-carboxy-tetramethyl-rhodamine (TAMRA); p: phosphorylation.

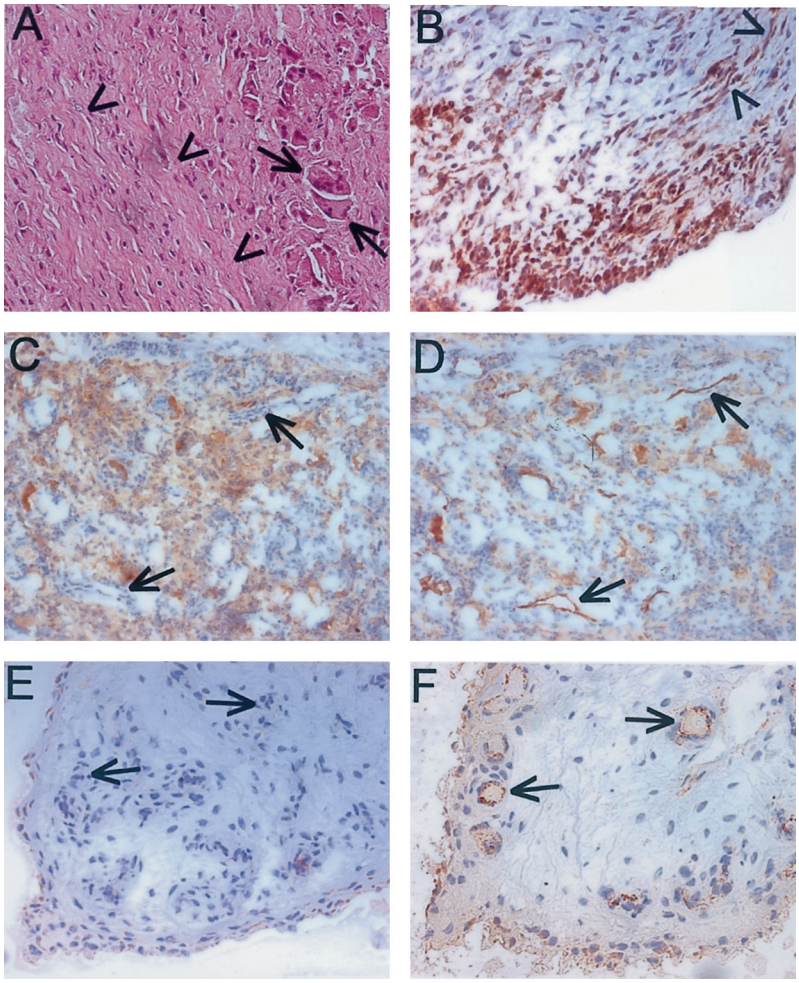

Figure 1. Histological and immunocytochemical analysis of synovial membrane-like interface tissue. (A) H&E staining of interface tissue. Stroma of the interface tissue consists of cell-rich areas that contain macrophage-like cells, multinuclear giant cells, and fibroblasts. Multinuclear giant cells (arrows) are often associated with large acetabular cup-derived polyethylene particles. Particle-rich areas are often surrounded by dense fibrous areas with dense collagenous fibers and scattered fibroblast-like cells (arrowheads). (B) Synovial membrane-like interface tissues display strong cathepsin K immunoreactivity in lining cells and in fibroblasts in the dense fibrous area (arrowheads). (C) Presence of RANKL is often associated with particles and multinuclear cells. Empty spaces within the tissue result from polymethylmethacrylate particles, which are dissolved during tissue processing. RANKL protein is lacking in the vascular structures (arrows). (D) OPG immunoreactivity in the same area as in panel C. OPG is mainly detected in the vascular endothelium (arrows) and is much weaker or absent in other areas. (E) Control synovial membrane from patient in surgery due to femoral neck fracture displays weak RANKL immunoreactivity in lining cells but not in vascular structures (arrows). (F) OPG is present in vascular endothelium (arrows) and in lining cells in control synovium. The displayed area is the same as in panels C and D (interface tissue) and E and F (control synovial membrane).

samples and was often associated with wear debris particles (Figure 1C). In contrast, OPG protein was present in vascular endothelium, but was mainly absent in the areas where RANKL was present (Figure 1D). Synovial membrane derived from patients who underwent surgery due to femoral neck fracture displayed weak RANKL immunoreactivity within the lining cell layer (Figure 1E). As in interface tissue, OPG was detected mainly in vascular endothelium. Some OPG immunoreactivity was also present in lining cells (Figure 1F).

Phenotype of the cells. All cells derived from the interface tissue of loose hip replacement prostheses and from the hip joint capsule showed strong immunoreactivity with fibroblast marker 5B5 antibody. No cultures contained endothelial cells, monocytes, or smooth muscle cells as revealed by the corresponding antibodies against the phenotypical markers of these cells (data not shown). Osteoblast phenotype was confirmed by observing that after longterm culture they started to form mineralized nodules, as visualized with von Kossa staining (data not shown).

RANKL Western blotting. Western blotting revealed a 35 kDa membrane-bound RANKL protein in the positive control osteoblast lysates in addition to the interface tissue fibroblast and fibrous capsule fibroblast lysates (Figure 2). A protein of similar size was present in giant cell tumor lysate. The antibody also recognized recombinant human 28 kDa soluble RANKL protein.

OPG ELISA. Basal and stimulated production of OPG protein was relatively high in osteoblast culture media (Figure 3A), and increased 2-fold upon TNF-α stimulation ($p < 0.001$) in osteoblast cultures and 1.5 fold ($p < 0.001$) in interface tissue fibroblast cultures. Fibroblast cultures from fibrous capsule also increased their OPG release upon TNF-α stimulation ($p < 0.05$); however, the amount always remained 1.5 to 2-fold lower than that of interface tissue fibroblasts. OPG seems to be rapidly secreted, since amounts of it in all cell lysates were extremely low (data not shown).

RANKL ELISA. The amount of RANKL protein in all cell lysates was always at least 10 times lower than that of OPG in culture media. Surprisingly, the amount of RANKL protein was highest in unstimulated fibroblast lysates from interface tissue. There were no statistically significant differences between fibroblasts of different origin or different

stimulations, although the amount of RANKL was always higher in interface tissue than in fibrous capsule fibroblasts. Fibroblastic RANKL production was very similar to that of osteoblasts (Figure 3B). The highest RANKL-OPG ratios were always seen in nonstimulated cultures for all cell types studied (Figure 3C). The low RANKL level in lysates of stimulated cells was not an artefact caused by solubilization of cell membrane-bound RANKL as ELISA of the cell culture supernatant did not reveal any measurable concentrations of RANKL.

Quantitative RT-PCR for RANKL and OPG. Both RANKL and OPG mRNA were expressed in interface tissue fibroblasts (Figure 4). In osteoblast and fibroblast cultures, OPG mRNA production was quite similar. There was a statistically significant correlation between the amount of secreted OPG protein and OPG mRNA in osteoblast cultures ($r = 0.83$, $p < 0.001$), interface tissue fibroblast cultures ($r = 0.42$, $p < 0.001$), and fibrous capsule fibroblast cultures ($r = 0.73$, $p < 0.001$). The expression of RANKL mRNA was very low in all cell types and stimulations. The expression was highest in fibrous capsule fibroblast cultures. The highest RANKL mRNA levels were measured after 1α,25-$(OH)_2D_3$ stimulation, but even in such cultures RANKL protein in the cell lysates remained relatively low. No statistical differences were seen between different stimulations or cell types studied in the RANKL mRNA levels analyzed. The RANKL-OPG mRNA ratio was extremely low. The number of RANKL mRNA copies was 25,000 to 60,000 times lower than that of OPG mRNA in osteoblasts, and 10,000 to 20,000 times lower in interface tissue and fibrous capsule fibroblast cultures, respectively. The RANKL-OPG mRNA ratio was lowest in osteoblast cultures. As for the corresponding protein levels, the RANKL-OPG mRNA ratios were usually highest in nonstimulated cells (Figure 4).

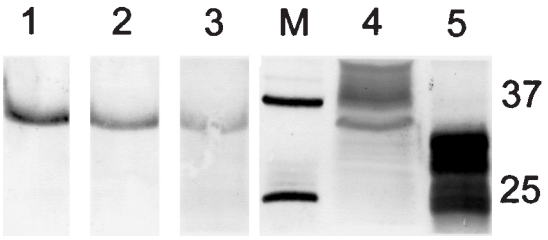

Figure 2. Western blot analysis of RANKL. Lanes 1–3: 35 kDa RANKL protein in the lysates of osteoblasts, interface tissue fibroblasts, and fibrous capsule fibroblasts. Lane M: molecular weight marker with 37 kDa and 25 kDa bands indicated on the right. Lane 4: 35 kDa RANKL protein in a giant cell tumor of bone lysate. Lane 5: Recombinant 28 kDa soluble human RANKL lacking the transmembrane part of the molecule, and thus running faster than the native RANKL protein.

Cathepsin K Western blotting. Fibroblasts can produce and release cathepsin K protein. This was not regulated by any of the soluble factors we investigated. There were no clear differences in the amounts of 42 kDa pro-cathepsin K or 27 kDa active cathepsin K released by the stimulated and nonstimulated cultures (Figure 5).

Quantitative RT-PCR for cathepsin K. Unstimulated fibroblasts produced 9 ± 1 cathepsin K mRNA copies per one PBGD copy. Fibroblasts increased their cathepsin K mRNA production to 31 ± 10 copies ($p < 0.01$) after 1α,25-$(OH)_2D_3$ stimulation. TNF-α (9 ± 1 mRNA copies) and IL-1ß (9 ± 1

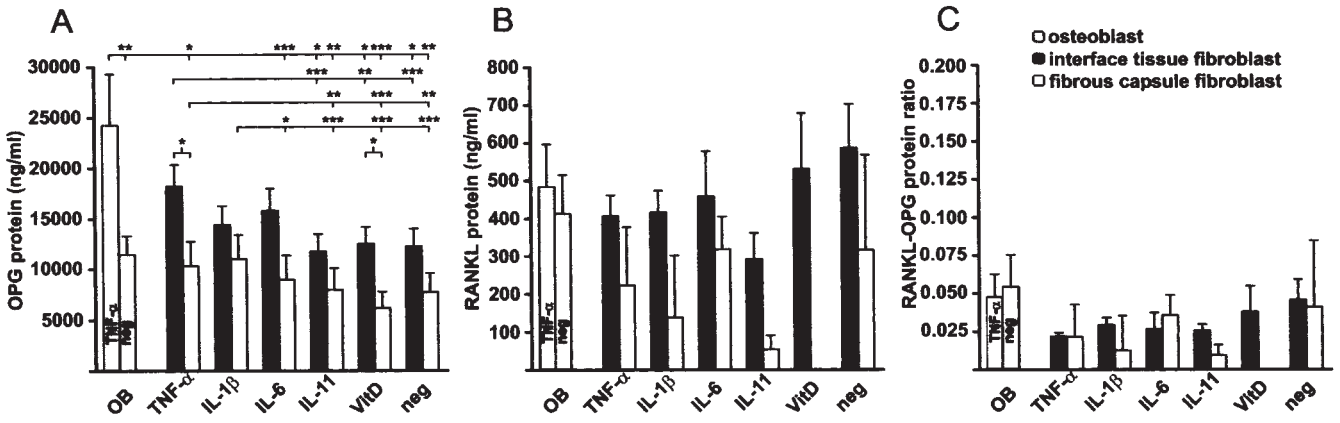

Figure 3. ELISA measurements of OPG in culture media after cytokine and 1α,25-$(OH)_2D_3$ stimulations. The first 2 bars represent osteoblast cultures stimulated and unstimulated with TNF-α. (A) OPG production by TNF-α stimulated osteoblasts was greater than that of nonstimulated osteoblasts, interface tissue fibroblasts (black bars), or fibrous capsule fibroblasts (white bars) with p values as indicated. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Interface tissue fibroblasts stimulated with TNF-α produced higher levels of OPG than those stimulated with IL-11 ($p < 0.001$), 1α,25-$(OH)_2D_3$ ($p < 0.01$), or nonstimulated cells ($p < 0.001$). TNF-α stimulated fibrous capsule fibroblasts produce higher levels of OPG than IL-11 ($p < 0.01$), 1α,25-$(OH)_2D_3$ stimulated ($p < 0.001$), or nonstimulated ($p < 0.01$) cells. IL-1ß stimulation significantly increased fibrous capsule fibroblast OPG compared to IL-6 ($p < 0.05$) and IL-11, 1α,25-$(OH)_2D_3$ stimulated or nonstimulated cells ($p < 0.001$). Interface tissue fibroblasts produced more OPG than fibrous capsule fibroblasts after TNF-α and 1α,25-$(OH)_2D_3$ ($p < 0.05$). (B) The amount of RANKL protein in cell lysates. RANKL concentrations were at least 10-fold lower than those of OPG in the corresponding culture media. (C) RANKL-OPG ratios in the stimulated cell cultures. There were no significant differences in RANKL-OPG ratios within or between the groups. The ratio indicated is RANKL concentration divided by OPG concentration measured from the same cultures.

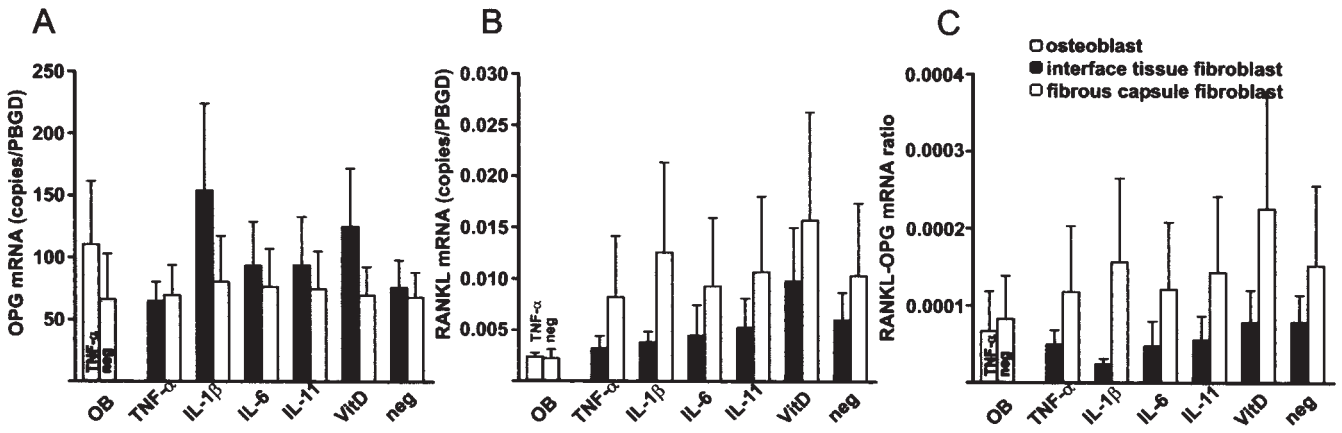

Figure 4. Cellular OPG and RANKL mRNA expression after cytokine and 1α,25-$(OH)_2D_3$ stimulations. The first 2 bars represent the TNF-α stimulated and nonstimulated osteoblast cultures. The mRNA expression was normalized to housekeeping gene PBGD mRNA expression. (A) OPG mRNA and (B) RANKL mRNA values. RANKL mRNA copy numbers were at least 10,000-fold lower than the corresponding OPG mRNA copy numbers. (C) RANKL-OPG ratios of the cultures. Black bars represent values at interface tissue fibroblasts and white bars the values of fibrous capsule fibroblasts.

mRNA copies) did not alter fibroblast-mediated cathepsin K mRNA production. Cathepsin K mRNA production was slightly increased after IL-6 (13 ± 4 mRNA copies) and IL-11 (17 ± 5 mRNA copies) stimulations, but the increase was not statistically significant.

DISCUSSION

Osteoclast formation in periprosthetic bone is likely to be mediated, at least in part, by the RANKL system[6-9]. Binding of RANKL to RANK expressed on the surface of osteoclast precursor cells induces cell fusion and osteoclast activation. The decoy receptor OPG blocks this process. Pathologically high OPG level generates osteopetrosis and lack of OPG leads to osteoporosis[13,20-22]. It seems that RANKL and OPG as well as cathepsin K, a major bone collagenolytic protease[23], may play central roles in aseptic loosening of total hip replacement prostheses[6-9].

We observed that fibroblasts are the source of RANKL in the interface tissue. Synovial fibroblasts from rheumatoid arthritis samples have also been shown to promote bone destruction by supporting osteoclast formation via their RANKL production[18,24]. In the current study, Western blots showed a clear 35 kDa immunoreactive RANKL band in cell lysates of interface fibroblast cultures. The molecular weight corresponding to the band is similar to that reported in the literature[25,26].

Production of RANKL in osteoblasts is regulated by various cytokines. In this study TNF-α, IL-1ß, IL-6, or IL-11 did not affect RANKL production at the mRNA or protein level in fibroblasts. However, this was also the case in osteoblasts. The only factor that consistently increased RANKL mRNA production in fibroblasts was 1α,25-$(OH)_2$ vitamin D_3. These findings suggest that RANKL production is not only cytokine but also cell type-dependent. Several studies have shown that mature osteoblasts decrease their RANKL synthesis and increase their OPG production during phenotype differentiation[27,28], and lose their ability to induce RANKL expression in response to stimulation[28,29]. This might be due to the CpG methylation around the transcription start site at RANKL gene, which is the case at least in mouse, and may cause the diversity of the stromal/osteoblastic cells in RANKL gene expression[29]. Although all cell types used in our study were early passage cells, they all originated from elderly patients, in whom the respective promoter region is probably strongly methylated. It is concluded that in contrast to, for example, embryonic or fetal tissue fibroblasts, fibroblasts from elderly individuals display certain basal expression of RANKL mRNA that is not affected by cytokines used in this study.

In contrast to RANKL, OPG was effectively regulated by the selected cytokines and hormones. As also shown in previous studies[30,31], TNF-α had a strong stimulatory effect on both OPG mRNA and protein levels in osteoblasts and in interface tissue fibroblasts. There was a positive correlation between OPG mRNA and protein levels. Interface tissue fibroblasts and fibrous capsule fibroblasts did not differ in their OPG mRNA levels; however, after TNF-α and 1α,25-$(OH)_2$ vitamin D_3 stimulations there were significant differences in the level of secreted OPG between fibroblasts from these 2 different sources. It seems that fibroblasts derived from interface tissue are primed for enhanced OPG protein synthesis, perhaps as an attempt to counteract osteolysis in loosening of THR. It can therefore be concluded that the RANKL-OPG ratio in aseptic loosening may be regulated by OPG rather than RANKL synthesis. Proinflammatory cytokines increased OPG production without an apparent effect on RANKL production in cultures of interface and capsular tissue fibroblasts. Based on RANKL production it seems that interface tissue fibroblasts, fibrous capsule fibroblasts, and osteoblasts can all generate the osteoclastogenic environment. However, local macrophage accumula-

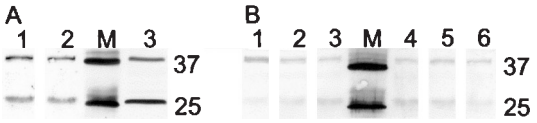

Figure 5. Representative Western blots show 42 kDa pro-cathepsin K and 27 kDa active cathepsin K secretion from fibroblasts. (A) Lane 1: culture medium sample from nonstimulated fibrous capsule fibroblasts; lane 2: culture medium from nonstimulated interface tissue fibroblasts; M: molecular weight marker with 37 kDa and 25 kDa bands marked on the right; and lane 3: control giant cell tumor of bone lysate. (B) Synovial membrane-like interface tissue fibroblasts release similar amounts of pro- and active cathepsin K into the cell culture medium with and without stimuli. Culture media from lane 1: TNF-α stimulated; lane 2: IL-1ß stimulated; lane 3: IL-6 stimulated; lane 4: IL-11 stimulated; lane 5: 1α,25-$(OH)_2D_3$ stimulated; and lane 6: nonstimulated fibroblasts. Lane M: molecular weight marker with 37 kDa and 25 kDa bands indicated on the right.

tion, a prerequisite for pre-osteoclast fusion, is a result of particle-mediated chronic inflammation. The RANKL-OPG ratio was very low in all cell types studied, and after different stimulations. Based on this, it seems that RANKL-induced macrophage/pre-osteoclast fusion is limited by high avidity binding of RANKL to OPG. This further implies that for effective osteoclast formation, a direct cell to cell contact between RANKL-expressing fibroblast or osteoblast and macrophage/osteoclast progenitor cells expressing RANK is probably required in interface tissue. Indeed, Takahashi and coworkers have shown that such a contact appears to be necessary for effective osteoclastogenesis to occur[32]. However, it cannot be excluded that in inflammatory conditions *in vivo* the situation may be different. Based on immunocytochemical observations OPG is absent in the areas where RANKL is present, and thus in such areas RANKL may be able to interact directly with RANK without interference from OPG. Although immunocytochemistry revealed that RANKL is diffusely distributed, it is not clear whether soluble or membrane-bound RANKL is more important in inducing osteoclast fusion in the interface tissue. Our recent study[33] indicates that secreted soluble RANKL is present in pseudosynovial fluids derived from patients with aseptic loosening of THR prostheses. Pseudosynovial fluid was found to be capable of inducing the formation of bone-resorbing multinuclear cells from peripheral blood mononuclear cells *in vitro*[33]. This indicates that both soluble and membrane-bound RANKL may induce cell fusion within the THR interface tissue.

Cells in the THR peri-implant tissue are affected by multiple factors able to modulate cellular activity[34]. IL-1ß and TNF-α may also induce osteoclast formation and bone resorption activity in the absence of RANKL. Based on our results, it seems that in the interface tissue the effect of IL-1ß and TNF-α on osteoclastogenesis is not necessarily mediated by their effect on RANKL production in fibroblasts. They more likely have an effect on macrophages and osteoclasts directly.

One of the main proteases for collagenolysis in bone is the acidic endoproteinase cathepsin K that degrades collagen type I at several sites in the triple helical region[23,35]. It has been thought that only macrophages, osteoclasts, and foreign body giant cells produce cathepsin K. We found that fibroblasts of both noninflammatory and inflammatory tissue produce and release cathepsin K. Although the role of other proteinases, such as matrix metalloproteinases, in periprosthetic collagenolysis cannot be ruled out, the production of cathepsin K may be especially important in interface tissue, in which the low pH is optimal for cathepsin K activity[6]. Western blots indicated that the production of cathepsin K was not affected by the studied cytokines; however, quantitative RT-PCR results showed that 1α,25-$(OH)_2$ vitamin D_3 stimulation increased cathepsin K mRNA levels. It has been shown that cathepsin K production in rheumatoid synovial fibroblasts[36] is elevated by TNF-α and IL-1ß stimulation[37]. Therefore, rheumatoid fibroblasts may be particularly aggressive in this respect.

This is the first investigation in which RANKL, OPG, and cathepsin K were studied simultaneously at the mRNA and protein level in interface tissue fibroblasts and with a broad set of cytokines and 1α,25-$(OH)_2D_3$. We conclude that fibroblasts are able to produce RANKL, OPG, and cathepsin K. The results indicate that interface tissue fibroblasts may have a potential to control osteoclastogenesis during the loosening of total hip replacement prostheses. However, based on these results it seems that blocking osteoclastogenesis by inhibition of RANKL would not prevent cathepsin K production in the interface tissue. Further studies are needed to determine if particles, micromotion, and changes in fluid pressure and shear stress, which are important regulators of fibroblast function, may also modulate interface tissue fibroblast RANKL, osteoprotegerin, and cathepsin K production.

ACKNOWLEDGMENT

Janica Wakkinen, Pauliina Porola, and Robert Björkenheim are greatly appreciated for their expert technical help.

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