Imbalance of RANKL/RANK/OPG system in interface tissue in loosening of total hip replacement

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In the differentiation of osteoclasts the differentiation factor (RANKL) interacts with the receptor activator of NF-κB (RANK) in a direct cell-to-cell contact between osteoblast and (pre)osteoclast. This is inhibited by soluble osteoprotegerin (OPG). The mRNA levels of both RANKL (p < 0.01) and RANK (p < 0.05) were high in peri-implant tissue and RANKL+ and RANK+ cells were found in such tissue. Double labelling also disclosed soluble RANKL bound to RANK+ cells. We were unable to stimulate fibroblasts to express RANKL in vitro, but monocyte activation with LPS gave a fivefold increase in RANK mRNA levels. In contrast to RANKL and RANK expression in peri-implant tissue, expression of OPG was restricted to vascular endothelium. Endothelial cell OPG mRNA levels were regulated by TNF-α and VEGF, but not by hypoxia. It is concluded that activated cells in the interface tissue overproduce both RANKL and RANK and they can interact without interference by OPG.

Aseptic loosening in total hip replacement (THR) is always characterised by the formation of synovial membrane-like interface tissue between the implant and host bone. Foreign bodies produced in a metal-to-plastic gliding surface migrate to this interface membrane and cause a chronic, macrophage-dominated foreign-body reaction. This is associated with periprosthetic bone lysis, but the mechanisms responsible are not yet known. Similarly, it is not known how activation affects fibroblast-, macrophage- and endothelial-cell-mediated production of some of the key molecules regulating the differentiation of osteoclasts (OC).

The development and function of OC involve multiple tightly-controlled stages. OC precursors differentiate to mononuclear prefusion OC, fuse to multinuclear OC and finally start to resorb bone. It had already been hypothesised that there must be some kind of OC differentiation factor on the surface of the osteoblast since formation of OC was not possible without co-culture of osteoblasts and monocytes. This factor was first found on the surface of activated T-cells and named tumour necrosis factor (TNF)-related activation-induced cytokine (TRANCE) or receptor activator of NFκB ligand (RANKL). This protein was later found also to be a membrane-bound osteoblast and fibroblast cytokine which regulated the differentiation of OC. It was isolated by utilising binding to osteoprotegerin and has therefore also been named osteoprotegerin ligand (OPGL). Characterisation of this factor confirmed its essential role in the differentiation and activation of OC.

Differentiation begins when RANKL (OPGL/TRANCE) binds to RANK, which is expressed on the surface of precursors of OC and mature OC. Blocking of RANKL with soluble osteoprotegerin (OPG), also known as osteoclastogenesis-inhibitory factor (OCIF), inhibits the formation of OC. In vitro OPG diminishes the formation of OC in a dose-dependent manner. In vivo OPG causes severe osteopetrosis both after intravenous administration or in overexpression studies.

Over 20 different cytokines which regulate bone resorption/OC and bone formation/osteoblasts have been studied and are held to be in part responsible for peri-implant bone loss. This together with cyclic mechanical loading and micromovement finally leads to aseptic loosening. More recently, it has been suggested that all these cytokines mediate most of their effects on bone metabolism indirectly via
the RANKL-system, which would also make the components of this system amenable targets for the prevention of pathological bone loss. This led us to investigate the eventual presence and the effect of activation of the producer cells on the key molecules involved in the normal development of OC, namely RANKL, RANK and OPG, in fibroblasts, macrophages and endothelial cells, respectively, in vivo in synovial membrane-like interface tissue and in vitro in cell cultures.

Patients and Methods

We studied 11 patients with aseptic loosening who were in pain and whose radiographs showed the presence of periprosthetic osteolysis. The mean time between the primary and revision operations (life in service) for the implants was 14.5 ± 1 (SEM) years. Samples for microbiological cultures were taken at each operation to confirm the aseptic nature of the loosening process and synovial membrane-like interface tissue was collected. Synovial membrane tissue from six patients undergoing a primary total hip replacement because of a fracture of the femoral neck was used as a control. All samples were snap frozen and stored at -70°C.

Cell culture. Monocytes were isolated from the blood-cell fraction of healthy blood donors of the Finnish Red Cross using the Ficoll-Paque PLUS (Pharmacia Biotech, Uppsala, Sweden) density-gradient technique. After washing the cells they were cultured in a 24-well plate at 4 x 10^6 cells/well in 1 ml of Macrophage serum-free medium (GibcoBRL, Paisley, Scotland) supplemented with 1% penicillin/streptomycin. After one hour, the cells were washed and the adherent cells were further cultured for 24 hours with or without 10 µg/ml of lipopolysaccharide (LPS) to activate macrophages. After this, the cells were washed once with phosphate-buffered saline (PBS) before isolation of RNA.

Human foreskin microvascular endothelial cells (hMVEC) were isolated, cultured and characterised as previously described.13 The hMVEC were cultured on gelatin-coated dishes in M199 supplemented with 20 mmol/l HEPES (pH 7.3), 10% human serum 10% heat-inactivated newborn calf serum, 150 µg/ml of crude ECGF, 2 mmol/l L-glutamine, 5 U/ml of heparin, 100 IU/ml of penicillin and 100 µg/ml of streptomycin at 37°C under 5% CO_2. Experiments were performed with confluent cells (0.7 x 10^5 cells/cm^2) which had been cultured without growth factors for at least 24 hours. The hMVEC were stimulated with TNFα (10 ng/ml), or VEGF_165 (25 ng/ml) (a gift from Dr H. Weich; GFB, Braunschweig, Germany) and TNFα (10 ng/ml) at normoxic (20% O_2) or hypoxic conditions (1% O_2 in the surrounding air). For culture in hypoxic conditions, the hMVEC were placed in a NAPCOÒ incubator, serial number 7101-C1 (Precision Scientific Inc, Chicago, Illinois), which controls the oxygen concentration by flushing with N_2. Oxygen levels in the incubator were monitored by an internal oxygen sensor as well as by external calibration using Dräger Tubes 6728081 (Drägerwerk Ag, Lübeck, Germany). After 24 hours, the total RNA was isolated.

Human fetal skin fibroblasts were cultured in RPMI medium (Biomedicum Helsinki) supplemented with 10% human serum and 1% penicillin/streptomycin. Cells were grown to confluence after which a new media with supplements and 4 nM phorbol myristate acetate (PMA) (Sigma, St Louis, Missouri) were added to the cells. They were stimulated for 24 hours and subjected to isolation of RNA.

Immunohistochemistry. Cryostat sections (6 µm) were mounted on DAKO Capillary Microscope slides (TechMate; Dako, Clostrup, Denmark), fixed in cold acetone for five minutes at 4°C, and washed in 0.01 M PBS (pH 7.4) for five minutes. The slides were then washed with washing buffer and installed in a DAKO TechMate Horizon immunostainer and stained automatically at 22°C using the following protocol:

1) the primary antibody, diluted with DAKO ChemMate antibody diluent, for 25 minutes; the concentrations of the antibodies were goat anti-human RANK IgG, 0.25 µg/ml, mouse anti-human RANKL IgG_2B 1 µg/ml, and mouse anti-human OPG IgG_1/κ, 1.25 µg/ml (R & D Systems, Minneapolis, Minnesota); 2) biotinylated secondary antibody for 25 minutes; 3) block peroxidase for 25 minutes; 4) peroxidase-conjugated streptavidine three times for three minutes; 5) HRP substrate buffer; and finally, 6) substrate working solution containing 3,3’-diaminobenzidine tetrahydrochloride (ChemMate detection kit) for five minutes. Between each step, the sections were washed with DAKO ChemMate washing buffers three times and dried in absorbent pads. After staining, the sections were removed from the machine, counterstained with haematoxylin, washed, dehydrated in ethanol series, cleared in xylene and mounted. Normal goat IgG or monoclonal mouse IgG_2b/κ or IgG_1/κ of irrelevant specificity were used at the concentration as and instead of the primary antibodies as negative-staining controls.

For double immunofluorescence, staining sections were fixed in cold acetone for 20 minutes at -20°C. After fixation, sections were incubated with normal donkey serum (Vector Laboratories, Burlingame, California), diluted 1:20 in PBS containing 1.25% BSA for 30 minutes at 22°C followed by blotting excess normal serum. RANK and RANKL antibodies were the same as those used for immunohistochemistry as described above. Mouse anti-human CD68 1gG_1 was purchased from DAKO. RANK was then labelled using TRITC-conjugated donkey anti-goat IgG and RANKL with FITC-conjugated donkey anti-mouse IgG (RANKL/RANK double staining). All secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania) and diluted 1:100 in PBS containing 12.5% BSA and 0.8 g/l of human IgG to reduce non-specific staining. After washing, the sections were air-dried and mounted with Vectashield mounting medium (Vector Laboratories).

Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated by the use of TRizol reagent (Gibco). Messenger RNA (mRNA) was iso-
lated from total RNA using magnetic (dT)$_{25}$-polystyrene beads (Dynal, Oslo, Norway) with a PickPen magnetic particle collector (BioNobile, Turku, Finland). We used 100 ng of mRNA to prepare primary cDNA using (dT)$_{12-18}$ primers and SuperScript enzyme, followed by RNase H treatment (GibcoBRL). Quantitative PCR was run on 5 ng of first-strand cDNA using 0.5 mM primers and 0.2 mM TaqMan probes (Table I) in LightCycler PCR mix by a LightCycler PCR machine (Roche Molecular Biochemicals, Mannheim, Germany). The identity of the product from one sample of each group and from the positive control was verified from 50 ng isolated amplicon (QIAquick; Qiagen Inc, Chatsworth, California) using an automated Applied Biosystems 373 A sequencer. Probes had reporter dye FAM (6-carboxyfluorescein) at the 5´ end and quencher dye TAMRA (6-carboxy-tetramethyl-rhodamine) at the 3´ end, which was also phosphorylated. Serial 1:10 dilutions of human genomic DNA were used to determine the copy number of the amplicon per 1000 $\beta$-actin mRNA copies. The $\beta$-actin copy numbers were similar in all samples and therefore it was possible to use $\beta$-actin as a standard gene and a marker for successful synthesis of cDNA. All primers were designed so that they 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 $\beta$-actin to exclude the possibility of genomic DNA contamination. The results were analysed by a Mann-Whitney independent rank-sum test performed with the BMDP New System statistics program (BMDP Statistical Software Inc, Los Angeles, California).

### Results

**RANKL, RANK and OPG detection by immunohistochemistry.** RANK protein was found in interface tissue, particularly in multinuclear foreign-body giant cells (Fig. 1a). By contrast, synovial membrane from patients with fracture of the femoral neck did not display any cells staining for RANK (Fig. 1b). RANKL protein was also found in interface membranes (Fig. 1c), but not in synovial membrane tissue from patients with fracture of the femoral neck (Fig. 1d).

OPG protein was found in both synovial membrane-like interface tissue and synovial membrane from patients with

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### Table I. Sequences of primers and probes used in quantitative PCR. Gene and corresponding sequence accession number are shown. Primers are designed and tested so that they are inside the exon in all genes

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<th>3 primer</th>
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*6-carboxy-fluorescein (FAM), x, 6-carboxy-tetramethyl-rhodamine (TAMRA); p, phosphorylation
†probes anneal to minus strand except RANK probe, which anneals to plus strand and is marked with dash

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Fig. 1

Photomicrographs of biotin-streptavidin-peroxidase staining (counterstained with haematoxylin) of synovial membrane-like interface tissue of loosened THR samples and synovial membrane of trauma samples. Both RANK (A) and RANKL (C) show strong immunoreactivity in the interface tissue, but not in the trauma samples (B, D). By contrast, staining showed OPG only in vascular endothelium in both the synovial membrane-like interface in aseptic loosening of a THR (E) and in the synovial membrane in trauma patients (F) (× 200).
fracture of the femoral neck. OPG staining was restricted to vascular endothelial cells whereas macrophage-like cells, foreign-body giant cells and fibroblasts were OPG-negative (Figs 1e and 1f).

Double immunofluorescence staining for RANK and RANKL disclosed both double and single positive cells (Fig. 2). mRNA expression level analysis by quantitative RT-PCR. Quantitative RT-PCR revealed that synovial membrane-like interface tissue contained more RANKL and RANK mRNA copies than synovial tissue from patients with fracture of the femoral neck. Interface tissue samples contained a mean of 6 ± 1 copies of RANK mRNA whereas synovial membrane samples had only 3 ± 1 copies (p < 0.05). The difference in RANKL was even more significant 18 ± 3 vs 5 ± 2 (p < 0.01). By contrast, the mean OPG mRNA level was the same in both groups (41 ± 11 vs 41 ± 10 copies) (Fig. 3).

Cell-culture studies for RANK, RANKL and OPG. The cell-culture experiments showed that cultured peripheral blood monocytes (n = 2) produced a mean of 52 ± 9 copies of RANK mRNA after LPS stimulation compared with 11 ± 1 copies without LPS stimulation. Production of OPG mRNA in human endothelial cells was not dependent on the oxygen level, but cytokine stimulation affected production of OPG. Non-stimulated hMVEC cells (n = 4) produced less than 2 ± 1 OPG mRNA copies whereas TNFα-stimulated hMVEC cells produced 9 ± 0.05 copies (p < 0.01) and TNFα/VEGF costimulated hMVEC cells produced 10 ± 0.1 copies (p < 0.01) of OPG mRNA against 1000 copies of β-actin mRNA. The results were independent of the oxygen level. Human fetal skin fibroblasts stimulated with phorbol myristate acetate (PMA) did not produce detectable levels of RANKL mRNA.

Discussion
A normal patient with a THR takes about 10⁶ steps per year and at each step over 10⁵ polyethylene particles alone are formed in a conventional low-friction metal-to-plastic gliding pair. Wear debris leads to a chronic, foreign-body-type inflammation and to local production of at least 20 different cytokines, growth factors and interleukins, which are able to stimulate OC. In addition, they have many other effects leading to activation of fibroblasts and endothelial cells, which are not directly activated by phagocytosis of foreign bodies. Our aim was to assess if this foreign-body-initiated inflammation cascade leads to the upregulation of the final common pathway of OC activation, the RANKL/RANK/
OPG system. We found that 1) RANKL and RANK are upregulated at the mRNA and cellular (protein) level in stroma around loosening implants; 2) that OPG was localised to another tissue compartment (vascular endothelial cells) suggesting that it was not able to interfere with the direct contacts between RANKL+ and RANK+ cells and 3) that this RANKL/RANK interaction was in part confirmed by double staining, which disclosed RANKL bound to RANK+ cells. The balance between RANKL and OPG is important in the regulation of OC.10,16 Our findings indicate that soluble OPG had not been able to prevent interactions between solubilised RANKL and its cell-bound receptor RANK. These results are compatible with the hypothesis that the effect of the other OC cytokines could be mediated via the cell-bound and solubilised RANKL, which interact with RANK on macrophages/preosteoclasts.7,8,17,18 Normally, osteoblasts are responsible for the development of OC, but synovial fibroblasts derived from rheumatoid arthritis tissue produce RANKL and are able to stimulate their formation.19 Our results suggest that RANKL in interface tissue can stimulate RANK+ responder cells to differentiate to OC. It is likely that the RANK+ cells may develop to OC and are monocytes or macrophages since it has been shown that human peripheral blood monocytes and macrophages purified from interface tissue stimulated with RANKL become multinucleated osteoclastic bone-resorbing cells.20-22

Although the potential stimuli able to upregulate RANKL, RANK and OPG production in interface tissue are undoubtedly numerous, it was possible to make some interesting observations in this respect. First, it was found that, in spite of the presence of both RANKL and RANK mRNA in our control (trauma) samples, immunohistochemical staining did not disclose any corresponding proteins. This may have some trivial explanations such as too low sensitivity of the detection method. This, however, seems unlikely because both RANKL and RANK were shown to be present in the synovial membrane-like interface tissue, which was processed and fixed similarly to the control samples. In addition, the same antibodies and staining methods were used for labelling of both interface and control tissue samples. Therefore it must be concluded that RANKL and RANK mRNA is not translated to the corresponding mature proteins in normal synovial membranes. This could be due to a post-transcriptional control mechanism or there may be a threshold level for mRNA below which no protein is synthesised. Although the exact regulatory mechanism remains unclear, it is likely to be important and deserves further study.

The interface membrane contained increased levels of RANKL mRNA and immunohistochemical staining showed the presence of RANKL protein. In osteoblasts 1α25(OH)2-dihydroxyvitamin D3, prostaglandin E2,13 prostaglandin E1, and parathyroid hormone23 stimulate the production of RANKL. The effect of TFGβ is the opposite.16 As already mentioned, numerous cytokines and combinations thereof may explain the increased RANKL mRNA and protein levels in interface tissue. In our study, protein kinase C activator phorbol myristate acetate (PMA) was used to activate human foreskin fibroblasts, but it did not stimulate fibroblasts to produce any more RANKL mRNA than had already been produced by unstimulated fibroblasts. We conclude that at least the fibroblast-mediated RANKL production in interface tissue is hardly protein kinase C-dependent and may be relatively strictly regulated to avoid accidental development and activation of OC.

The level of RANK mRNA was increased in the interface membrane and immunohistochemical staining showed RANK in the tissue. As an in vitro model of macrophage activation, we used LPS or endotoxin, which is the principal component of the outer membrane of Gram-negative bacteria and to which monocytes are exquisitely sensitive. LPS binds to LPS-binding protein LBP in plasma and is then delivered to cell surface CD14 initiating a cascade, which leads to activation of NF-κB, three MAPK, ERK-1 and -2 etc.23 Monocyte activation effectively increased RANK mRNA copy numbers in monocytes.

The interface membrane contained OPG mRNA and immunohistochemical staining showed OPG to be present in endothelial cells. It has been shown to be essential to the endothelial cells as an anti-apoptotic cytokine25 and OPG-deficient mice have been shown to develop medial calcification of the aorta and renal arteries.26 OPG mRNA levels were similar in interface membrane and in the control samples. Because so many cytokines, growth factors and interleukins are produced in the interface, it was thought that endothelial cell-mediated OPG is perhaps constitutive. In contrast to this hypothesis, it was found that TNFα, with or without VEGF, caused a five-fold increase in human endothelial cell OPG mRNA levels. It has been shown that TNFα and IL1β increase the production of endothelial cell OPG and RANKL.27 We can confirm the effect of TNFα on the production of OPG in endothelial cells and further conclude that VEGF does not have any effect on the TNFα stimulus. Although it has been shown that RANKL is expressed in endothelial cells in vitro27 we were not able to show any RANKL protein in endothelium in vivo. The reason may be that OPG has bound to RANKL in endothelium and thus blocked the protein-antibody interaction. Interface tissue is active in which oxygen consumption is relatively high. Furthermore, since the medullary artery is destroyed in the course of a THR interface, tissue is hypovascular and subjected to ischemia-reperfusion.28 Accordingly, very low pH values have been measured per-operatively in revision operations.29 However, hypoxia did not stimulate the endothelial cells to produce more OPG mRNA and did not modulate the production of OPG mRNA induced by TNFα ± VEGF.

Our data are similar to the findings of Haynes et al30 which showed that RANK and RANKL are involved in wear-particle-induced formation of OC in periprosthetic tissue. Their study was performed in vitro with the cells
extracted from interface tissue and RNA analysis was carried out. We are able to support their in vitro data and have shown that RANK and RANKL mRNA is upregulated in vivo and that the RANK and RANKL proteins occur in the interface tissue. Haynes et al also found that particle and LPS stimulation can induce RANK and surprisingly RANKL mRNA expression in monocytes in vitro. We are able to confirm the increase of RANK mRNA after LPS stimulation, but we do not yet have enough data to confirm the increase in RANKL mRNA expression. Interestingly, the data of Haynes et al also show that cells from tissues containing silastic particles are more aggressive against bone than those from tissues containing metal or polyethylene particles. Based on these findings, it can be concluded that foreign bodies activate cells in the interface tissue to overproduce both RANKL and RANK, which can interact in the absence of OPG. This interaction could explain the differentiation of macrophages to multinuclear, TRAP and cathepsin-K-positive OC-like cells. Prevention of the production of RANKL or blockage of the RANK/RANKL interaction in interface tissue may help to increase the lifetime of a total hip replacement.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References


