

Imbalanced expression of RANKL and osteoprotegerin mRNA in pannus tissue of rheumatoid arthritis

M. Ainola^{1,2,3}, J. Mandelin^{3,4}, M. Liljeström³, Y.T. Konttinen^{1,2,4}, J. Salo⁵

¹Department of Medicine, Helsinki University Central Hospital, Helsinki; ²ORTON Orthopaedic Hospital of the Invalid Foundation, Helsinki; ³Department of Anatomy, University of Helsinki, Helsinki; ⁴COXA Hospital for the Joint Replacement, Tampere; ⁵Department of Orthopaedics and Traumatology, Helsinki University Central Hospital, Helsinki, Finland.

Abstract

Objective

To test if the pannus tissue is characterized by a high receptor activator of nuclear factor κ B ligand to osteoprotegerin (RANKL:OPG) ratio, which could explain local osteoclastogenesis and formation of bony erosions.

Methods

Messenger RNA and protein expressions of RANKL and OPG in rheumatoid and osteoarthritic tissue samples were measured using quantitative real-time RT-PCR and Western blot/densitometry. Pannus and synovitis fibroblasts explanted from tissue samples were cultured in vitro without and with TNF- α , IL-1 β or IL-17 and analyzed quantitatively for RANKL expression. The ability of pannus fibroblasts to induce formation of multinuclear osteoclast-like cells from human monocytes, with macrophage-colony stimulating factor (M-CSF) but without RANKL added, was tested. Histochemical staining was used to assess the eventual presence of RANKL and tartrate resistant acid phosphatase positive osteoclast-like cells at the pannus-bone interface.

Results

RANKL:OPG ratios of messenger RNA ($p < 0.05$) and protein level were high in pannus (2.06 ± 0.73 and 2.2 ± 0.65) compared to rheumatoid (0.62 ± 0.13 and 1.31 ± 0.69) and osteoarthritis (0.62 ± 0.32 and 0.52 ± 0.16) synovial membranes. Resting and stimulated (p dependent on the cytokine used) pannus fibroblasts produced RANKL in excess ($p = 0.0005$) and unstimulated pannus fibroblasts also effectively induced osteoclast-like cell formation from monocytes in vitro without any exogenous RANKL added. Compatible with these findings, multinuclear osteoclasts-like cells were frequent in the fibroblast- and macrophage-rich pannus tissue at the soft tissue-to-bone interface.

Conclusions

The high RANKL:OPG ratio, together with close fibroblast-to-monocyte contacts in pannus tissue, probably favor local generation of bone resorbing osteoclasts at the site of erosion in rheumatoid arthritis.

Key words

Rheumatoid arthritis, pannus, bone resorption, osteoclasts, RANKL.

Mari Ainola, MSc; Jami Mandelin, PhD;
Mikko Liljeström, BSc; Yrjö T. Konttinen,
MD, PhD; Jari Salo, MD, PhD.

The work should be attributed to the
Department of Medicine, Helsinki
University Central Hospital, Helsinki,
Finland; ORTON Orthopaedic Hospital
of the Invalid Foundation and Department
of Anatomy, University of Helsinki,
Helsinki, Finland.

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Please address correspondence and
reprint requests to: Mari Ainola, MSc,
Biomedicum Helsinki, Institute of
Biomedicine, Anatomy, P.O. Box 63
(Haartmaninkatu 8), University of
Helsinki, FIN-00014 Finland.
E-mail: mari.ainola@helsinki.fi

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Introduction

Rheumatoid arthritis (RA) is characterized by synovitis and pannus formation. Pannus is the leading edge of the RA synovial membrane, which grows into cartilage and bone. While invading into joint cartilage/bone, pannus is able to secrete extracellular matrix destroying enzymes *e.g.*, matrix metalloproteinases, cathepsins and mast cell proteinases, which have been demonstrated in RA pannus (1-5).

In contrast to cartilage destruction, bone resorption starts with osteoclast-mediated demineralization of bone matrix. Osteoclasts are formed from haematopoietic monocytes and macrophages by fusion. First osteoclast precursors differentiate to mononuclear pre-fusion cells, fuse to form multinuclear osteoclasts and finally are capable of resorbing bone. Indeed, multinuclear osteoclast-like cells have been implicated in bone destruction in RA (3, 6). Osteoclasts have been produced by co-culture of bone cells or peripheral blood monocytes with cells isolated from rheumatoid tissue in the presence of vitamin D₃ and macrophage-colony stimulating factor (M-CSF) (7, 8) or spontaneously from RA synovial cells (9), which implicates that synovial cells are capable of forming osteoclasts. Also, several regulatory cytokines enhance bone resorption by stimulating osteoclast-inducing factors (10).

Receptor activator of nuclear factor kappa B ligand (RANKL) is a member of TNF superfamily expressed on activated T-cells, osteoblast/stromal cells and chondrocytes (11). Previous studies have shown RANKL also in rheumatoid synovial tissue (12), cultured RA synoviocytes (8) and at the sites of bone erosion in collagen-induced arthritis (13-15). The absence of RANKL expression in knockout mice prevents osteoclast formation and subsequent bone erosion in inflammatory arthritis (16, 17). Recently, Pettit *et al.* showed induced RANKL expression in RA tissue at the site of erosions (18). These findings might implicate that fibroblast RANKL plays an important role also in the leading edge of pannus in RA.

Osteoprotegerin (OPG) is a decoy receptor for RANKL, which appears on

osteoblasts upon their activation (19), but is also found in endothelial cells and macrophages in synovial tissue and lining (20). OPG has been shown to block RANKL expression and consequently also to inhibit osteoclast formation and erosions in collagen- and adjuvant-induced arthritis (21, 22). The RANKL: OPG balance is therefore essential for the regulation of osteoclast formation. Recent studies have reported increased sRANKL and decreased OPG levels in synovial fluid (23).

Several papers have also described the balance of OPG and RANKL in RA patient sera, synovial fluid and/or synovitis tissues (20, 24-27). The new aspect in this study is its focus on pannus tissue and explanted pannus fibroblasts. This location is critical for the formation of erosions. We wanted to extend these earlier studies by analysing RANKL and OPG at the messenger RNA (qRT-PCR) and protein (Western blot and densitometry) level for the indication of this aggressive tissue involvement in local osteoclastogenesis in rheumatoid arthritis.

Materials and methods

Patients and samples

All tissue samples were collected from well-characterized rheumatoid arthritis (RA) and osteoarthritic patients, who fulfilled the American College of Rheumatology (ACR) criteria. Nine synovial membrane and eight pannus tissue samples were collected in parallel from advanced RA patients (8 women and 1 men) undergoing primary total hip replacement, knee arthroscopy or synovectomy. Pannus samples were taken from the site of invasion into cartilage/bone and synovium from further site of invasion. In one patient, the pannus sample did not contain soft-to-hard tissue junction and this pannus sample was disregarded. The mean age of these patients was 54 years (range 34-80 years). Nine synovial membrane samples were collected from osteoarthritic patients (8 women and 1 men) undergoing primary total hip replacement, with a mean age of 73 years (range 58-85 years). For RNA extraction, samples were during operation immediately snap frozen in pre-cooled isopentane and stored at -70°C

Competing interests: none declared.

or for immunohistochemistry samples were processed to paraffin by fixation in 10% formalin followed by dehydration in ethanol, clearing in xylene and embedding in paraffin. None of the patients included in the present study had any biological anti-inflammatory medications or any clinical or microbiological signs of infection. The sample collection was approved by the Ethics Committee of Helsinki University Central Hospital and the guidelines of the Declaration of Helsinki were followed.

Cell culture

Pannus and synovial fibroblasts from rheumatoid arthritis patients were established using explant culture method. Briefly, tissue samples were minced to pieces and left over night in RPMI-1640 medium (BioWhittaker, Liege, Belgium) containing 10% fetal bovine serum (FBS) (BioWhittaker) and 10% penicillin/streptomycin. The next day, the media was changed and the concentration of antibiotics was decreased to 1%. The media was changed twice a week and when 60% of the dish area was covered with a monolayer of cells, the tissue pieces were removed and the cultures were allowed to grow to confluence. The cells were characterized with immunofluorescence staining of fibronectin and vimentin, used at passages 3-5 and counted with Z1 Coulter Particle Counter (Beckman Coulter). For stimulations, cells were cultured to confluence in a 6 well plates at 1×10^5 cells per well. Two parallel wells were used for RNA extraction and one well with 4 coverslips for histochemistry. The cells were stimulated with 0.1, 1 and 10 ng/ml rhTNF- α , rhIL-1 β or rhIL-17 (R&D, Minneapolis, MN) for 48 hours. All fibroblast stimulations were made in triplicate.

Peripheral blood mononuclear cells were isolated from fresh buffy coats obtained from healthy blood donors of the Finnish Red Cross by the use of Ficoll-Paque PLUS (Pharmacia Biosciences, Uppsala, Sweden) density gradient. After washing, the cells were cultured in a 6-well plates at 5×10^6 cells per well in 2 ml of MEM Alpha Medium with Glutamax-1 (Invitrogen, Paisley, Scotland) supplemented with

10% FBS and 1% penicillin/streptomycin. After one hour, the non-adherent cells (containing predominantly lymphocytes) were removed and the adherent monocyte/macrophage-enriched cultures were further stimulated as fibroblasts or used for co-culture where cultured fibroblasts were added together with 40 ng/ml rhM-CSF (R&D) and with or without rhTNF- α , rhIL-1 β , rhIL-17 (R&D) or rhRANKL (Alexis, Carlsbad, CA, USA).

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Total RNA from frozen tissue (80 mg) and cultured cells (2 x 3.5 cm wells) were isolated by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. After that mRNA was isolated from total RNA using magnetic (dT)₂₅-polystyrene beads (Dyna, Oslo, Norway). 100 ng of mRNA was used to prepare primary cDNA using (dT)₁₂₋₁₈ primers and SuperScript enzyme, followed by RNase H treatment (Invitrogen). To ensure the correct synthesis, negative controls without RT enzyme or sample mRNA and positive control with control RNA were performed. Quantitative PCR was run on 3 ng of first strand cDNA using 0.5 mM primers for OPG [GenBank:U94332]: sense 5'-ggcattctcaggttgcgttccta-3' and antisense 5'-agctgtgtgcccgtttatcctctct-3', RANKL [GenBank:AF019047]: sense 5'-ccaacattgcttcgacatcatgaa-3' and antisense 5'-tgaccaataactgtgtgcttctct-3' and β -actin [GenBank:M10277]: sense 5'-tcaccacactgtgccatctacga-3' and antisense 5'-cagcgggaaccgctcattgccaatgg-3' with 0.2 mM TaqMan probes for OPG 5'-matttgctggcaccacaaagtaacgcaxp-3', RANKL 5'-macgtactaaaacagcatcaaaatcccaaxp-3' and β -actin 5'-matgcctccccatgccatcctgcgtp-3' in LightCyclerTM PCR mix by LightCyclerTM PCR machine (Roche Molecular Biochemicals, Mannheim, Germany). Probes anneal to minus strand and had reporter dye FAM (6-carboxyfluorescein) at the 5' end and quencher dye TAMRA (6-carboxy-tetramethylrhodamine) at the 3' end, which was also phosphorylated. For primers and probes, corresponding sequences were

searched from NCBI Entrez search system, sequence similarity search was done using NCBI blastn program and were produced by Proligo (Paris, France). All primers were designed to be located inside one exon for the possible use of genomic DNA as a standard. The identity of the product was verified by size using PCR amplification (Dyna, Finnzymes) and by sequence from 50 ng of isolated (QIAquick, Qiagen) amplicon on automated Applied Biosystems 373 A sequencer. Serial 1:10 dilutions of human genomic DNA were used to determine the mRNA copy numbers of the amplicons per 1000 β -actin for tissue and 100,000 for cell culture samples. Each individual sample was amplified at least two times for all genes of interest.

Histo- and cytochemistry

Serial 3 μ m thick paraffin tissue sections were mounted on DAKO Capillary Microscope slides (TechMateTM, Dako, Glostrup, Denmark) and dried overnight. After deparaffinization, the slides were washed with washing buffer and placed in DAKO TechMateTM Automated Immunostainer 500 and processed at 22°C using DAKO ChemMateTM Reagent System and MSiPE protocol with 30 minutes enzymatic digestion in 4 mg/ml pepsin for antigen retrieval and an extra 30 minute incubation with primary antibody. The concentrations of the antibodies were as follows: 10 μ g/ml for mouse anti-human RANKL IgG_{2b} (R&D) and the same concentration for the negative control mouse IgG_{2b} (Dako). Endogenous peroxidase activity was blocked with peroxidase solution. Staining was visualized using peroxidase-conjugated streptavidine and hydrogen peroxide and diaminobenzidine (DAB). For immunofluorescence, the tissue samples were incubated for 30 minutes in 10 μ g/ml primary mouse anti-human RANKL IgG_{2b} (R&D) and the same concentration for the negative control mouse IgG_{2b} (Dako) followed by 30 minutes in secondary Alexa Fluor 594-conjugated goat anti-mouse IgG (Molecular Probes, Leiden, Netherlands). In co-cultures, the multinuclear osteoclast-like cells were stained for tartrate

resistant acid phosphatase (TRAP) with leukocyte acid phosphatase kit (387-A, Sigma, Steinheim, Germany). Nuclei were stained with 4',6-Diamidino-2-phenylindole (DAPI) (Sigma).

Western blotting

Synovium (n=3) and pannus (n=3) of RA and synovium of osteoarthritis (n=3) were homogenized in RIPA Buffer Set (Boehringer Mannheim, Germany), sonicated, centrifuged and filtrated. Electrophoresis was performed using 75 µg total protein, blotted onto nitrocellulose membrane, blocked overnight in 3% bovine serum albumin (BSA) and incubated for 90 minutes with 2 µg/ml monoclonal anti-human OPG IgG_{2a} (R&D) or 0.2 µg/ml biotinylated polyclonal rabbit anti-human RANKL IgG (PeproTech) in 2% BSA. Detection was performed using alkaline phosphatase-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) or alkaline phosphatase-conjugated avidin (Sigma) with colour development solution (Alkaline Phosphatase Conjugate Substrate Kit, Bio-Rad, Hercules, CA). Optical densities of the bands were analyzed with Phoretix 1D software (Nonlinear dynamics).

Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was performed using GraphPad Prism version 3.02 for Windows (GraphPad Software Inc., San Diego, CA). Paired *t*-test was used for comparing paired samples between two groups. One-way ANOVA was used for multiple group comparisons combined with Dunnett's *post-hoc* test to compare against control column and Bonferroni's to compare all columns.

Results

RANKL mRNA expression is increased in rheumatoid pannus tissue

Quantitative RT-PCR revealed that pannus tissue contained more RANKL mRNA copies than rheumatoid or osteoarthritic synovium ($p=0.03$) (Fig. 1). Pannus tissue samples (n = 8) contained 73.3 ± 14.5 copies of RANKL mRNA whereas RA synovial membrane samples (n=9) contained 53.4 ± 10.9

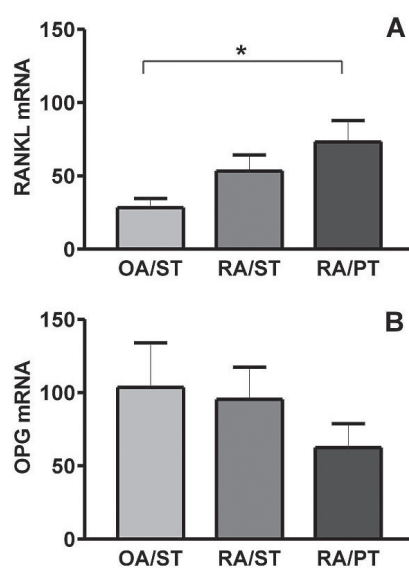


Fig. 1. RANKL and OPG expressions in tissue samples. (A) RANKL and (B) OPG mRNA copy numbers (normalised for β-actin) in osteoarthritic synovial tissue (OA/ST), rheumatoid arthritic synovial tissue (RA/ST) and pannus tissue (RA/PT). * $p < 0.05$.

RANKL mRNA copies. The expression of RANKL mRNA in OA synovium (n=9) was low: 28.3 ± 6.3 copies ($p < 0.05$). In contrast, the expression of OPG mRNA was highest in OA samples. In pannus the OPG mRNA level was 62.5 ± 16.3 . RA synovium samples contained 95.5 ± 21.8 OPG mRNA copies and OA samples 103.5 ± 30.5 . In pannus tissue the RANKL mRNA production is higher than OPG production (73.3 ± 14.5 vs. 62.5 ± 16.3). In OA the RANKL mRNA expression was almost four times lower than OPG expression (28.3 ± 6.3 vs. 103.5 ± 30.5) and in RA

almost two times lower (53.4 ± 10.9 vs. 95.5 ± 21.8). The mRNA expressions of RANKL, OPG or the RANKL:OPG ratio did not correlate with the age of the patients (data not shown).

Increased expression of RANKL in pannus tissue was also confirmed at the protein level by using Western blot and densitometric measurements (Fig. 2). In pannus tissue samples (n=3), the average intensity of RANKL band was 72.7 ± 14.7 compared to lower intensity in RA synovium 41.4 ± 16.1 and lowest intensity in OA synovium 40.6 ± 14.3 . Also OPG protein showed same kind of pattern as mRNA expression. Highest intensity was found in OA synovium 79.1 ± 13.3 and lowest in pannus tissue 34.7 ± 3.3 . The intensity of OPG in RA synovium was intermediate 45.4 ± 13.1 .

Rheumatoid pannus fibroblasts express RANKL

Fibroblasts established from rheumatoid pannus and synovitis tissue contained RANKL mRNA. Interestingly, pannus fibroblasts express more RANKL than synovitis fibroblasts ($p = 0.0005$). Stimulations of both pannus and synovitis fibroblasts with 0.1, 1 and 10 ng/ml TNF-α, IL-1β or IL-17 increased RANKL mRNA in a dose-dependent fashion, except pannus stimulation with TNF-α (Fig. 3). 10 ng/ml was the most effective dose and using this dose, the RANKL mRNA levels of pannus fibroblasts were 215-fold increased upon stimulation with TNF-α (32.29 ± 7.13 vs. 0.15 ± 0.05 , n=3) ($p < 0.001$), 18-fold

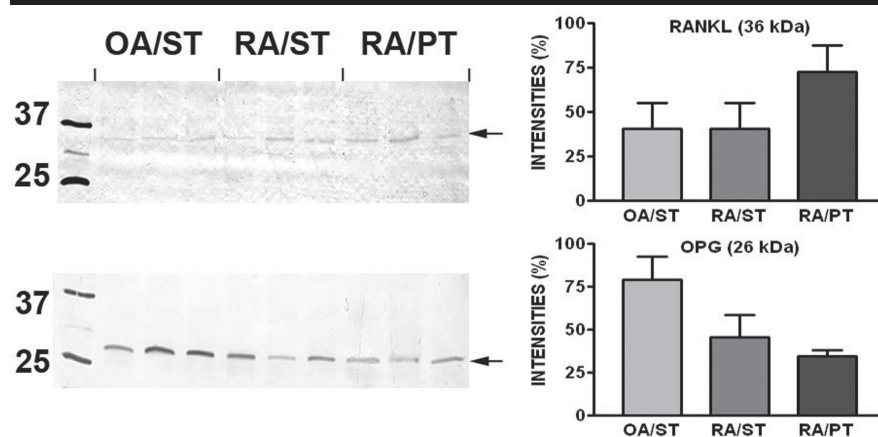


Fig. 2. Western blot analysis of RANKL and OPG in osteoarthritic synovial tissue (OA/ST), rheumatoid arthritic synovial tissue (RA/ST) and pannus tissue (RA/PT). Intensities of the bands are normalized to the most strongly expressed band and shown as mean values.

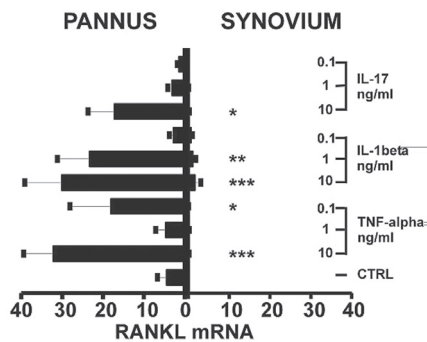


Fig. 3. RANKL expression in rheumatoid fibroblasts. RANKL mRNA copy numbers (normalised for β -actin) in proinflammatory cytokine stimulated pannus fibroblasts and synovitis fibroblasts. *** $p<0.001$, ** $p<0.01$, * $p<0.05$ difference of RA pannus to synovitis fibroblasts.

increased upon stimulation with IL-1 β (30.22 ± 8.97 vs. 1.71 ± 1.36 , $n=3$) ($p<0.001$) and 91-fold increased upon stimulation with IL-17 (17.31 ± 6.39 vs. 0.19 ± 0.05 , $n=3$) ($p<0.05$) compared to synovitis tissue fibroblasts. No mRNA expression could be detected in stimulated monocytes.

Pannus fibroblasts stimulate multinuclear cell formation

Co-culture of pannus fibroblasts and monocytes stimulated with M-CSF and with or without rhTNF- α , rhIL-1 β or rhIL-17 produced multinuclear cells after 14 day of culture. All of these cells were TRAP positive (Fig. 4). Most effective stimulants were rhTNF- α and rhIL-1 β , when compared to RANKL stimulated positive control.

RANKL is located in bone-soft tissue junction near multinuclear cells

RANKL staining was most intense in pannus compared to RA and osteoarthritic synovial tissues. Positive cells were found around pannus tissue and interestingly also located near bone-soft tissue junction (Fig. 5A). Immunofluorescence staining shows more clearly RANKL positive cells at pannus-bone interface (Fig. 5B). More focused analysis with double staining indicates RANKL staining near TRAP positive multinuclear cells in pannus tissue (Fig. 6).

Discussion

After cartilage has been degraded, pannus can continue invasion to subchondral bone (28). This phenomenon

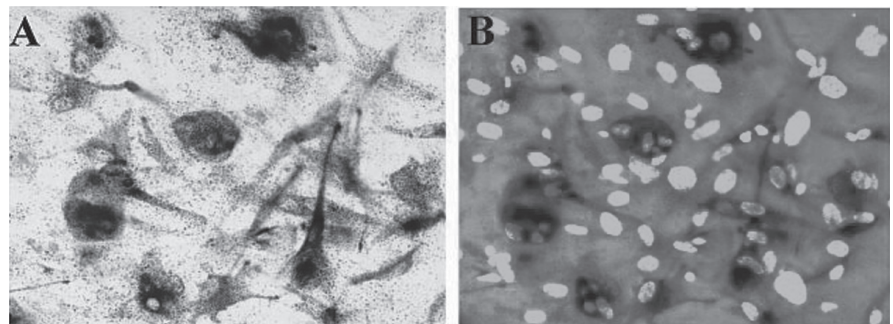


Fig. 4. Formation of multinuclear cells in co-culture. (A) TRAP staining for multinuclear osteoclast-like cells and (B) DAPI for nuclei in co-culture of pannus fibroblasts and monocytes stimulated with M-CSF and rhTNF- α . Original magnification x200.

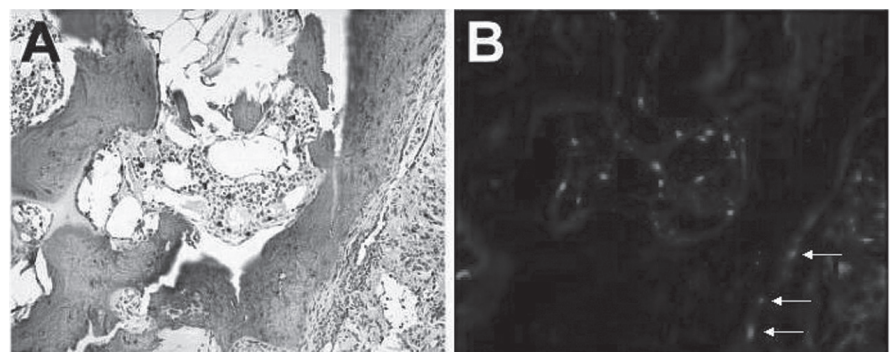


Fig. 5. RANKL immunoreactivity in pannus tissue samples. (A) Immunohistochemical staining of RANKL in pannus-bone interface and (B) serial section with immunofluorescence staining showing more specific RANKL expression near interface (arrows). Original magnification x100.

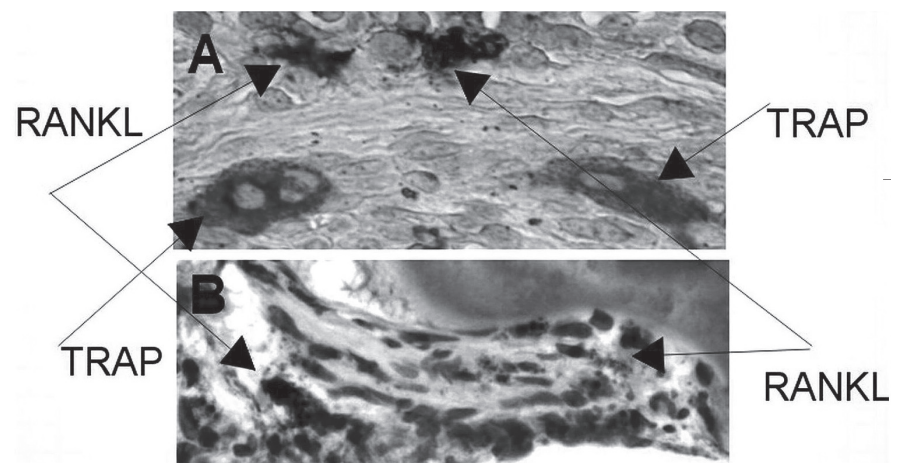


Fig. 6. Multinuclear cells in pannus tissue. Immunohistochemical (A) double staining for RANKL expression and TRAP for multinuclear osteoclast-like cells in pannus tissue and (B) staining for RANKL at the pannus-bone interface. Original magnification x400.

involves first secretion of pannus-derived proteinases for cartilage degradation, followed by local production of growth and differentiation factors that might stimulate formation and function of osteoclasts. This speculation is based on the fact that osteoclasts are the only cells, which can dissolve bone mineral

matrix with subsequent degradation of the demineralised organic type I collagen-rich matrix. Therefore, they probably play a role in the formation of bone erosions. RANKL seems to be the key factor stimulating osteoclast formation (11). If RANKL binds with its receptor RANK on the surface of monocyte/

macrophages, they fuse to form multinuclear giant cells. In remodelling bone tissue, RANKL positive osteoblasts are involved in osteoclast formation. Direct contact between osteoblast and precursor/prefusion osteoclast leads to formation of multinuclear bone resorbing cells. Although osteoblasts normally stimulate osteoclast formation in remodelling bone tissue, also synovial membrane fibroblasts produce and express RANKL and are able to stimulate osteoclast formation (29, 30). To be able to do so, RANKL expressed by both the osteoblast and fibroblast needs to come into contact with the RANK positive responder cells. If instead the cell surface (or solubilized) RANKL is bound by soluble decoy receptor OPG, its effect is inhibited (31). Therefore, the local balance between RANKL and OPG is decisive for the outcome, *i.e.*, whether osteoclasts are formed or not. Although RANKL production in synovitis tissue is certainly important, it seems logical to measure RANKL also in rheumatoid pannus tissue with respect to OPG levels. This work demonstrates that the RANKL:OPG ratios of both mRNA and protein are concordant and particularly high in rheumatoid pannus compared to rheumatoid arthritis synovitis tissue further away from the erosions or in osteoarthritis synovial membrane, a disease that is usually characterized by subchondral bone sclerosis and peripheral osteophytes, not erosions. Interestingly, not only was RANKL relatively high in RA, but OPG was rather low in RA, indicating that it is not only the positive driving force (RANKL), but also the negative breaking force of OPG that fails in pannus tissue. This indicates that the local milieu in rheumatoid arthritis is in favour for osteoclastogenesis. This conclusion was supported by demonstration of TRAP positive osteoclasts-like cells in the pannus-bone tissue interface as well as by the local formation of joint erosions.

As was mentioned above, osteoblasts initially were thought to play a primary role in osteoclast formation, but later the potential of the fibroblast to fulfil the same task has been recognized. *In vitro*, in the absence of OPG, both cells

can be replaced by soluble RANKL. It has been well established that peripheral blood monocytes can be stimulated to form osteoclasts by addition of RANKL together with M-CSF. In our experiments we demonstrate that in the presence of pannus fibroblasts human peripheral blood monocytes form multinuclear osteoclasts-like cells if only M-CSF is added. This suggests that pannus fibroblasts effectively provide RANKL in sufficient quantities to stimulate the RANK positive precursor/prefusion cells to fuse and form multinuclear osteoclasts-like cells. This confirms and extends the earlier findings reported by Haynes and coworkers (8). This hypothesis was further confirmed by quantitative RT-PCR, which demonstrated the presence of RANKL in pannus fibroblasts and that the RANKL mRNA levels were higher in pannus than in synovitis tissue fibroblasts. The local increase of RANKL expression is not only caused by increased numbers of RANKL positive cells in inflamed tissue, but is also caused by a greater production of RANKL per cell. This was shown by measuring the RANKL mRNA levels per housekeeping gene levels, which are expressed at the same level in all cells (and, therefore, called "housekeeping"). These values were shown to be increased in rheumatoid pannus compared to rheumatoid synovitis and OA synovium. In contrast, in protein level measurement, such "normalization" is not usually done so it is not possible to know if the higher amount of RANKL protein results from increased numbers of cells or greater production of RANKL per cell.

These findings extend earlier reports by Shunichi Shiozawa and his co-workers, which demonstrate that the leading edge of pannus tissue contains fibroblast-like cells (32, 33). Furthermore, pannus tissue shares many features of malignant mesenchymal tumor, so that a term "mesenchymal transformation" has been introduced to emphasize the potential role of fibroblasts in the formation of joint erosions (34, 35). Although activated T-cells have been earlier recognized to be RANKL positive (21) and able to stimulate osteoclastogenesis (23), we believe that, in

particular, the pannus fibroblasts play a key role in the local formation of osteoclasts at the pannus-bone tissue interface. Direct cellular contacts between pannus fibroblasts and monocyte/macrophages, in a similar fashion as has been described for osteoblasts and monocyte/macrophages (36, 37), promote osteoclast formation. Such direct cell-to-cell contacts may be necessary for RANKL-to-RANK interaction to conceal the site of this molecular interaction from the soluble OPG inhibitor.

Conclusion

Synovial inflammation leads in RA to formation of aggressive and tissue destructive pannus, which grows to cartilage/bone matrix. Based on our *in vivo* and *in vitro* findings we conclude that local fibroblast-mononuclear phagocyte co-operation and the high RANKL-OPG ratio drive local formation of osteoclasts. Such locally confined processes at the site of tissue destruction may explain ongoing and progressive formation of erosions even in the absence of systemic inflammation, *e.g.*, in the presence of normal erythrocyte sedimentation rate and C-reactive protein. Improved understanding of pannus invasion and degradation of bone matrix in rheumatic arthritis will probably open us new methods to prevent joint tissue destruction.

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