Synovial Fibroblasts Infected with *Salmonella enterica* Serovar Typhimurium Mediate Osteoclast Differentiation and Activation

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The mechanisms whereby arthritogenic organisms may induce cartilage and bone erosions in infection-triggered arthritis remain unknown. In this study, we asked whether an arthritogenic organism could contribute to osteoclast differentiation and activation through regulation of the receptor activator of NF-κB ligand (RANKL) in synovial fibroblasts. Rat synovial fibroblasts were infected in vitro with *Salmonella enterica* serovar Typhimurium and monitored over time. The expression of RANKL in resting and infected synovial fibroblasts was quantified by reverse transcription-PCR and Western blotting. Osteoclast progenitors, isolated from femurs of 8-week-old rats and cultured in the presence of macrophage colony-stimulating factor, were cocultured with either infected or noninfected synovial fibroblasts for 2 to 4 days. Differentiation and maturation of osteoclasts were determined by morphology and tartrate-resistant acid phosphatase (TRAP) staining and by a bone resorption bioassay. RANKL expression was undetectable in resting synovial fibroblasts but was dose-dependently upregulated in cells after *Salmonella* infection. Osteoprotegerin was constitutively expressed by synovial fibroblasts and was not upregulated by infection. Further, we observed the formation of multinucleated TRAP-positive cells and formation of bone resorption pits in cocultures of bone marrow-derived osteoclast precursors with synovial fibroblasts infected with *Salmonella* but not with heat-killed *Salmonella* or noninfected cells. Arthritogenic bacteria may alter bone structure via synovial fibroblast intermediaries, since infected synovial fibroblasts (i) upregulate RANKL expression and (ii) enhance osteoclast precursor maturation into multinucleated, TRAP-positive, bone-resorbing, osteoclast-like cells. These data provide a link between infection and osteoclastogenesis. A better understanding of infection-mediated osteoclast differentiation and activation may provide new therapeutic strategies for inflammatory joint disease.

Gastrointestinal infections by enteric bacteria such as *Salmonella* and *Yersinia* and urogenital infection by *Chlamydia* are known to be associated with reactive arthritis (ReA). While ReA in many cases follows a self-limited course, we found that of 27 patients with acute post-*Salmonella* ReA, 18 still had persisting disease activity at a 5-year follow-up (20). Post-*Salmonella* ReA can be accompanied by erosive sacroilitis (13). Despite the strong temporal association with antecedent infection, the pathogenesis of ReA is not resolved, but there is strong evidence of local persistence of microbial antigens (6) and microbial nucleic acids (4). Infection has been detected in neutrophils and macrophages for prolonged periods after the inciting infection, although synovial fibroblasts were not specifically examined (5). We have recently reviewed the several lines of evidence for the persistence of microbes or their products within the joints in ReA (21). Defining the initial phases of ReA has proved to be difficult in the clinical setting, and we used two approaches to analyze these host-pathogen interactions. We have demonstrated by using an in vitro system that synovial fibroblasts can be used to analyze direct host-pathogen interactions and that such cells can harbor low level of viable bacteria for up to 2 weeks (10). We have also demonstrated that synovial fibroblasts are appropriate target cells for arthritogenic *Chlamydia trachomatis* and that such infected cells can serve as a reservoir of local antigen in an animal model of ReA (9). In this model, the chronic aspecific arthritis is accompanied by significant erosion of cartilage and bone. The mechanisms by which these arthritogenic organisms might mediate bone destruction remain largely unknown.

Under normal circumstances, the maintenance of bone mass depends on a dynamic balance between bone resorption by osteoclasts and bone formation by osteoblasts. If, in the course of disease, the balance is tipped toward the osteoclast, bone resorption will exceed bone formation and the result will be bone destruction and loss (1). Among major regulators of differentiation and activation of osteoclasts are macrophage colony-stimulating factor (M-CSF), receptor activator of NF-κB ligand (RANKL), and osteoprotegerin (OPG) (19). M-CSF and RANKL induce osteoclast differentiation, maturation, and activation (osteoclastogenesis), leading to bone resorption, while OPG acts as a decoy receptor for RANKL and protects against bone destruction. It has been reported that RANKL is expressed by synovial fibroblasts derived from synovial tissues of patients with rheumatoid arthritis (RA) (7,
18), suggesting that synovial fibroblasts could contribute directly to the formation and activation of osteoclasts at sites of bone erosion in rheumatoid joints.

In the present study, we asked whether synovial fibroblasts with or without infection by an arthritogenic strain of *Salmonella enterica* serovar Typhimurium expressed RANKL or OPG and stimulated osteoclastogenesis from bone marrow precursors.

**MATERIALS AND METHODS**

**Isolation and culture of synovial fibroblasts.** The use of synovial fibroblasts for in vitro studies of arthritis pathogenesis has been described before (8, 12). The method that we used for generating synovocyte lines has been described previously (9). This method is an effective way of harvesting a substantial number of synovocytes from the joint. Briefly, an adult Lewis rat was immunized subcutaneously with methylated bovine serum albumin (mBSA) (Sigma Chemical Co., St. Louis, Mo.) in complete Freund’s adjuvant. Seven days later, the animal was injected subcutaneously with mBSA alone. One week later, the animal was injected into the knee joint with a sterile mBSA solution in phosphate-buffered saline (PBS). Forty-eight hours later, the animal was sacrificed, the knee joints were removed, and the inflamed tissue was removed. The tissues were minced and cultured as explant pieces in a 24-well plate. Within 14 days, fibroblast-like cells had migrated out from the tissue explants and formed confluent monolayers. The cells were collected by trypsinization and resedimented into flasks for expansion. Rat synovial fibroblasts prepared in this way could be passaged repeatedly and showed no sign of contact inhibition. These cells are stable in culture without any change of morphology or minimal essential medium containing 15% heat-inactivated fetal bovine serum.

**Bacterial infection of synovial fibroblasts.** A clinical isolate of *S. enterica* serovar Typhimurium recovered from a stool culture of a patient with post-Salmonella ReA was used in this study. The bacteria were cultured and enumerated as previously described (10) and then were added at predetermined ratios of bacteria to synovial fibroblasts in 12-well plates and left for 1 h. The fibroblasts were gently washed with the culture medium, and gentamicin was added at a concentration of 50 μg/ml to kill extracellular bacteria. After 1 h, cells were gently washed in culture medium to remove dead bacteria and then cultured in the presence of gentamicin at 10 μg/ml for 2 to 4 days. This concentration of gentamicin inhibits growth of any bacteria remaining in the culture medium but does not penetrate the cell membrane to kill intracellular bacteria (3, 10). Such infected synovial fibroblasts, or uninfected controls, were analyzed for the expression of RANKL and OPG and were utilized for coculture with the osteoclast precursors.

**Semiquantitative RT-PCR.** The expression of RANKL and OPG mRNAs in synovial fibroblasts was analyzed by semiquantitative reverse transcription-PCR (RT-PCR). UMR 106, a murine osteoblastic cell line, was used as positive control, since both RANKL and OPG mRNAs are expressed in this cell line (15). Total RNA was isolated from infected or noninfected synovial fibroblasts by using the Rneasy reagent (Invitrogen, Burlington, Ontario, Canada). Total RNA (1 μg) was reverse transcribed into first-strand cDNA by using a mixture of 1.0 μl of oligo(dT) (0.5 μg/μl), 2.0 μl of 10 mM deoxynucleoside triphosphates, 1.0 μl of 50 mM MgCl2, 1.0 μl of SuperScript II RNase H- reverse transcriptase (200 U/μl), 0.5 μl of Nase OUT recombinant RNase inhibitor (40 U/μl) (Invitrogen), 2.0 μl of 10× amplification buffer, and double-distilled water to bring the total volume to 20 μl. The mixture was incubated at 37°C for 30 min. PCR was performed with primers specific for rat RANKL, OPG, and the housekeeping gene L32. The PCR started with 4 μl of reverse transcription mixture (cDNA), 1.5 μl of 10× PCR buffer, 25 pmol of primers, and double-distilled water to bring the volume to 17.5 μl, with incubation at 95°C for 1 min. The temperature was held at 80°C in order to add 1.5 U of Taq DNA polymerase (1.0 μl; 5 U/μl) (Invitrogen). The PCR was continued for 22 cycles (94°C for 15 s, 55°C for 15 s, 72°C for 25 s, and a final elongation step at 72°C for 7 min) for L32, 30 cycles for OPG, and 30 cycles for RANK-L, with an annealing temperature of 61°C. Primers were as follows: OPG upstream, TTTGTGTGCAACAATGTGCC; OPG downstream, GAGCATCTGAGTGAAG; RANKL upstream, ACGAGATTTGCAACGTC; and RANKL downstream, TTCTGTGCTCCCTCTITTCATC (15).

**Western blotting.** The expression of RANKL and OPG proteins by synovial fibroblasts was analyzed by immunoblotting. Cell lysates of either infected or noninfected synovial fibroblasts were prepared, boiled for 10 min, and subjected to sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis with a mini-Protein 3 cell (Bio-Rad Laboratories, Hercules, Calif.). The proteins separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis were then transferred to a nitrocellulose membrane with a mini-Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories). Each membrane was incubated with primary antibody at 1:1,000 for RANKL (rabbit immunoglobulin G [IgG]; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.). 1:250 for OPG (goat IgG; Santa Cruz Biotechnology), and 1:5,000 for β-actin (mouse IgG; Sigma) in Tris-buffered saline–Tween 20 containing 5% fat-free milk. The membranes were washed and then incubated with, at 1:10,000, secondary antibodies of goat anti-rabbit IgG, rabbit anti-goat IgG, or goat anti-mouse IgG conjugated with horseradish peroxidase (Cedarlane Laboratories Limited, Hornby, Ontario, Canada). The membranes were analyzed by using ECL chemiluminescence (Amersham Biosciences, Piscataway, N.J.).

**Coculture of osteoclast precursors with synovial fibroblasts.** For harvesting of osteoclast precursors, we adapted the method of Quinn et al. (16) from the murine system to the rat. Total bone marrow containing osteoclast precursors was isolated from femurs of 8-week-old Lewis rats by flushing the long bone with culture medium (o-minimal essential medium containing 15% fetal bovine serum), and 106 nucleated cells were cultured in 1 ml of culture medium in T-75 tissue culture flasks. M-CSF at 2,000 U/ml was added in order to enrich the osteoclast precursor population. Three days later, cells from the nonadherent cell fraction were collected, counted, and added to synovial fibroblasts infected with live or heat-killed *Salmonella* or to noninfected cells in 12-well plates at a 10:1 ratio in culture medium containing M-CSF (1,250 U/ml), gentamicin (10 μg/ml), 1,25-dihydroxy-vitamin D3 (10−8 M), and prostaglandin E2 (10−6 M) for 2 to 4 days. The culture medium was replaced every 2 days.

**Osteoclast formation detected by TRAP staining and bone resorption assay.** To assess whether osteoclast precursors matured in coculture with synovial fibroblasts, cells were assessed microscopically for multinucleated osteoclasts on slides, by TRAP staining, and by a bone resorption assay. Cells were cocultured in 12-well plates, washed once with PBS, fixed with 3.7% formaldehyde for 5 min at room temperature, and then stained with, per well, 1.0 ml of freshly prepared TRAP staining solution containing naphthol-AS-MX phosphate sodium salt, N,N-dimethylylformamide, anhydrous sodium acetate, sodium tartrate (100 mM) and fast red TR salt (Sigma). After staining, the solution was aspirated and replaced with 1.0 ml of PBS. The TRAP-positive (red), multinucleated osteoclasts were visualized and counted under light microscopy. To assay bone resorption, bone marrow cells were cocultured with synovial fibroblasts in wells containing osteologic disks coated with submicron synthetic calcium phosphate thin films (BD Biosciences, Discovery Labware, Mississauga, Ontario, Canada). Cells were cultured for 2 to 4 days and removed by addition of 20% bleach. The disks were washed with distilled water and air dried. Resorption pits were visualized under light microscopy and quantified by image analysis (ImageJ, version 1.32a; National Institutes of Health, Bethesda, Md.).

**Statistical analysis.** Data were analyzed by analysis of variance (ANOVA) and Bonferroni post hoc tests with SPSS software (version 11.0). Statistical significance was taken as a P value of <0.05.

**RESULTS**

**OPG expression by synovial fibroblasts.** OPG mRNA (Fig. 1A) but not protein (not shown) was detected in both resting and *Salmonella*-infected synovial fibroblasts. Levels were similar in uninfected synovial fibroblasts and fibroblasts infected at either dose of bacteria tested (Fig. 1B). These results suggested that OPG mRNA is constitutively expressed by synovial fibroblasts and is not affected by *Salmonella* infection.

**Induction of RANKL expression by infected synovial fibroblasts.** RANKL expression was undetectable in resting synovial fibroblasts, but both mRNA (Fig. 1) and protein (Fig. 2) were easily detected in *Salmonella*-infected fibroblasts at 2 days and beyond. The ratio of bacteria to synovial fibroblasts influenced expression, since higher RANKL expression was observed in synovial fibroblasts treated with a higher *Salmonella*-to-fibroblast ratio (10:1 versus 5:1) by both RT-PCR and Western blotting (*P < 0.0001*) (Fig. 1 and 2). Thus, the RANKL/OPG ratio was increased in infected compared to uninfected synovial fibroblasts (*P < 0.0001*) (Fig. 1B), suggesting that infected cells may have an enhanced capacity to support osteoclastogenesis and bone resorption.
Infected synovial fibroblasts stimulate osteoclastogenesis and bone resorption. Numerous TRAP-positive, multinucleated mature osteoclast-like cells were seen in bone marrow cocultures with *Salmonella*-infected synovial fibroblasts (31 ± 6.6 TRAP-positive cells/cm²), but only a relatively few were seen with heat-killed *Salmonella* (1.9 ± 0.5 TRAP-positive cells/cm²) and resting synovial fibroblasts (1.8 ± 0.5 TRAP-positive cells/cm²) (Fig. 3). Consistent with this, resorption pits covered a larger fraction of the calcium phosphate disks in cocultures with infected fibroblasts (1.97%) than in those with uninfected fibroblasts (0.07%), which in turn was not different from the fraction seen with bone marrow cells alone (Fig. 4).

DISCUSSION

There is evidence clinically, in the case of post-*Salmonella* ReA (13), and experimentally, in the case of synovial fibroblast-packaged pathogens (9), that antecedent infection can set in motion a pathological sequence of events resulting in erosive arthritis, particularly of the sacroiliac joint. However, the re-

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**FIG. 1.** RT-PCR analysis of OPG and RANKL mRNA expression in synovial fibroblasts uninfected or infected with *Salmonella* at a bacterium/cell ratio of 5:1 or 10:1. A: Total RNA was extracted from uninfected or infected synovial fibroblasts at different times postinfection, and RT-PCR was performed with specific primers for RANKL, OPG, and ribosomal protein L32 as a control. UMR 106, an osteoblastic cell line, served as a positive control for OPG and RANKL expression. B: PCR products of OPG and RANKL were normalized against that of L32. Data are expressed as the means ± standard deviations from three independent experiments. RANKL expression was much more upregulated than OPG expression after infection of *Salmonella* (for RANKL versus OPG, *P* < 0.0001 by ANOVA and *P* < 0.01 [**] and *P* < 0.05 [*] by Bonferroni post hoc tests).
relationship between the microbial trigger and the resulting sacroiliitis is not known. Since tissue samples, particularly those from the sacroiliac joints, are difficult to study in the clinical setting, we have addressed this relationship in an in vitro system.

We found that resting synovial fibroblasts expressed OPG but not RANKL. This is of interest in light of the recent observation that human synovial fibroblasts also constitutively express OPG (11). However, infection with *S. enterica* serovar Typhimurium had no effect on OPG expression level but induced expression of RANKL mRNA and protein. The marked change in the ratio of RANKL to OPG between uninfected and infected cells suggests that under normal conditions synovial fibroblasts may be only weakly osteoclastogenic and that after infection they become potent stimulators of osteoclast formation. This is consistent with the fact that we found many more TRAP-positive, multinucleated cells and higher bone resorption in cocultures with infected synovial fibroblasts than in those with uninfected fibroblasts. Furthermore, we found that synovial fibroblasts infected with heat-killed *Salmonella* had no effect on formation of TRAP-positive cells (Fig. 3), suggesting that the necessary preconditions must involve live

![Image of Immunoblotting of RANKL expression](http://iai.asm.org/)

**FIG. 2.** Immunoblotting of RANKL expression in synovial fibroblasts uninfected or infected with *Salmonella* at a bacterium/cell ratio of 5:1 or 10:1. A: Total cell lysates were prepared at different times from synovial fibroblasts either uninfected or infected with *Salmonella* at the indicated *Salmonella*/synovial fibroblast ratio. Immunoblotting was performed with antibodies specific for RANKL or β-actin as a housekeeping control. B: RANKL expression was normalized against that of β-actin. Data are expressed as the means ± standard deviations from three independent experiments. Increased *Salmonella* infection resulted in a increase of RANKL expression in synovial fibroblasts (ANOVA, *P* < 0.0001; Bonferroni post hoc tests *P* < 0.0001 [***] [for bacterium/cell ratio of 10:1 versus 5:1]).
organisms. These data indicate that synovial fibroblasts are essential intermediaries linking infection with an arthritogenic organism and osteoclastogenesis, with bone erosion as a consequence. As stated above, synovial fibroblasts in RA have the capability of contributing to bone erosion (7, 18), but in RA, unlike ReA, the potential role of microbial triggers is unresolved.

One recent study of psoriatic arthritis has demonstrated that osteoclast precursors which arise from peripheral blood mononuclear cells can migrate to inflamed synovium and subchondral bone, where they are exposed to local synovial cells which have upregulated RANKL, as shown by immunohistochemical analysis. This process leads to osteoclastogenesis and bone erosion (17). As is the case for RA, the mechanism accounting for upregulation of RANKL in the psoriatic arthritis joint is unknown. Studies addressing the pathways which modulate RANKL expression have largely addressed this process in activated T cells (19), but the precise signaling steps behind upregulation of RANKL expression remain incompletely understood. The role of infection in this sequence has not been addressed. The recent study of Kubota et al. (11) demonstrated constitutive expression of OPG in RA human synovial fibroblasts, but RANKL was not addressed in that study.

FIG. 3. Osteoclast formation detected by TRAP staining. A: More multinucleated TRAP-positive (red) osteoclasts were formed in cocultures (day 4) between bone marrow cells and Salmonella-infected synovial fibroblasts (ratio of 10:1) than in cocultures with uninfected cells. B: Quantification of multinucleated TRAP-positive osteoclasts. The bars represent the mean values ± standard deviations for triplicate wells and are representative of those from three independent experiments.
Our studies were focused on the dynamic interaction of OPG, RANKL, and osteoclasts. It has been reported recently that synovial fibroblasts infected with *Salmonella* produce tumor necrosis factor alpha but that this expression is only transient (14). Future studies might address the range of cytokines generated in this system.

Our studies are the first to show robust expression of RANKL, with concomitant stimulation of osteoclastogenesis and bone resorption, by synovial fibroblasts after infection with an arthritogenic organism, *S. enterica* serovar Typhimurium. Our results also provide a model in which to define the molecular mechanism by which arthritogenic bacteria stimulate

**FIG. 4.** Formation of resorption pits. A: Resorption pits on osteologic disks in cocultures (day 4) of bone marrow cells with uninfected or *Salmonella*-infected synovial fibroblasts (ratio, 10:1). B: The resorption pit area was calculated as a percentage of the total disk. The bars represent the mean values ± standard deviations for triplicate wells and disks and are representative of those from three independent experiments.
osteoclastogenesis, which could lead to new therapeutic targets for blocking bone destruction in chronic arthritis.

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