The role of fibroblasts and fibroblast-derived factors in periprosthetic osteolysis

Ph.D. thesis

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2011

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ABBREVIATIONS

AB	antibody
Ang-1	angiopoietin 1
bFGF	basic fibroblast growth factor
cDNA	complementary DNA
CD11b	cluster of differentiation (cluster of designation) 11b
CD90	cluster of differentiation 90 = Thy-1 (a fibroblast marker)
CM	conditioned media
CM-IFM	conditioned media harvested from explant cultures of interface membrane
CM-NSy	conditioned media harvested from explant cultures of normal synovial tissue
CM-RASy	conditioned media harvested from explant cultures of rheumatoid synovial tissue
c-myc	oncogene
COX-1	cyclooxygenase 1
COX-2	cyclooxygenase 2
CO_2	carbon dioxide
Ct	threshold cycle
DMEM	Dulbecco's modified minimal essential medium
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FGF-R	fibroblast growth factor receptor
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-6-phosphate dehydrogenase housekeeping gene
hAngio-1	human Riboquant Multiprobe RPA template
hCK26	human Riboquant Multiprobe RPA template
hCK3	human Riboquant Multiprobe RPA template
hCK4	human Riboquant Multiprobe RPA template
hCR4	human Riboquant Multiprobe RPA template
hCR5	human Riboquant Multiprobe RPA template
hCR6	human Riboquant Multiprobe RPA template

IFM	interface membrane
IFM-Fb	interface membrane fibroblast
IFN-γ	interferon-γ
IL-1α	interleukin-1α
IL-1β	interleukin-1β
IL-1RI	IL-1 receptor type I
IL-4	interleukin-4
IL-6	interleukin-6
IL-8	interleukin-8
IP-10	interferon-γ-inducible 10-kDa protein = CXCL-10 (a CXC chemokine)
LIF	leukemia inhibitory factor
L32	housekeeping gene
mAb	monoclonal antibody
MCP-1	monocyte chemoattractant protein 1
M-CSF	macrophage colony stimulating factor
MMP-1	matrix metalloproteinase-1 (collagenase)
mRNA	messenger ribonucleic acid
NF-KB	nuclear factor kappa-light-chain-enhancer of activated B cells
NSy	fresh normal synovial tissue
OA	osteoarthritis
OPG	osteoprotegerin, osteoclastogenesis inhibitory factor (a decoy receptor of RANKL)
OSM	oncostatin M
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered salt solution
PCR	polymerase chain reaction
QRT-PCR	reverse transcription real-time quantitative polymerase chain reaction
RA	rheumatoid arthritis
RANKL	receptor activator of nuclear factor kappa-B ligand (osteoprotegerin ligand)
RANTES	regulated upon activation normally T-cell expressed and secreted (a CXC chemokine)
RASy	rheumatoid synovial tissue

rhRANKL	recombinant human RANKL
RNA	ribonucleic acid
RPA	RNase protection assay
SCF	stem cell factor
SDS	sodium duodecyl sulfate
SEM	standard error of the mean
SYBR Green	asymmetrical cyanine dye (a nucleic acid stain)
TGF-β1	transforming growth factor-β1
TGF-βRI	TGF-β receptor type I
Thy-1	thymocyte antigen 1 (a fibroblast surface marker)
Ti	titanium
TJA	total joint arthroplasty
TNF-α	tumor necrosis factor-α
TNFR p55	tumor necrosis factor receptor p55
TNFR p75	tumor necrosis factor receptor p75
TRAP	tartarate-resistant acid phosphatase
Untr	untreated
UTP	uridine 5'-triphosphate
VEGF	vascular endothelial growth factor
32P	phosphorus-32 (an isotope, used for labeling nucleic acids)

INTRODUCTION

Periprosthetic osteolysis following total joint arthroplasty (TJA) is a major clinical problem in both cemented and cementless reconstructions (**Fig. 1A**). Aseptic failure of total joint prostheses has emerged as the major clinical problem interfering with the long-term success of these arthroplasties. Factors that interact to produce aseptic loosening can be divided into several independent processes, including those that involve mechanical factors, the material properties of the implants, and biological and host factors.

One of the pathological features of periprosthetic osteolysis in failed TJAs is the formation of a pseudomembrane (interface membrane) at the bone/cement (**Fig. 1B**) or bone/prostheses interface (**Fig. 1C**). The importance of transformation of the material of an intact implant into particulate debris, and the capacity of the particles to induce a so-called foreign-body granulomatous response, is well established. Furthermore, the role of this tissue reaction in the inducement and perpetuation of peri-implant osteolysis has been recognized as a major problem contributing to aseptic loosening of total hip prostheses that have been inserted without cement. In fact, the hope that osteolysis would be eliminated was one reason for the development of implants that could be inserted without the use of cement.

Debris from total hip arthroplasties falls into three basic categories: polyethylene debris from the acetabular component, polymethylmethacrylate debris associated with implants that have been inserted with cement, and metal debris. The release of these materials in particulate form is responsible for the invasion of inflammatory cells and the formation of granulomas. Similar lesions have been produced by the introduction of poorly resorbable particles into other organs; for example, silicosis has developed in people who have been exposed to coal dust. In these conditions, the interaction of the particles with monocyte macrophages and inflammatory polykaryons results in the release of soluble inflammatory mediators. These mediators then act directly on host tissues or on local connective-tissue cells to produce alterations in tissue architecture and function. When this process occurs within skeletal tissue, the reaction may lead to a disturbance in bone-remodeling, manifested as osteolysis.

In addition to stimulating an inflammatory process, particulate debris can result in wear of other components, by increasing the amount of polyethylene particles that is generated, if the debris is caught between the articular surfaces (e.g. femoral head and the acetabulum).

Furthermore, metal debris may also have a toxic effect on cell function. This effect is mediated by the metal itself or by the corrosion or fretting corrosion products released by certain metals or metal alloys.

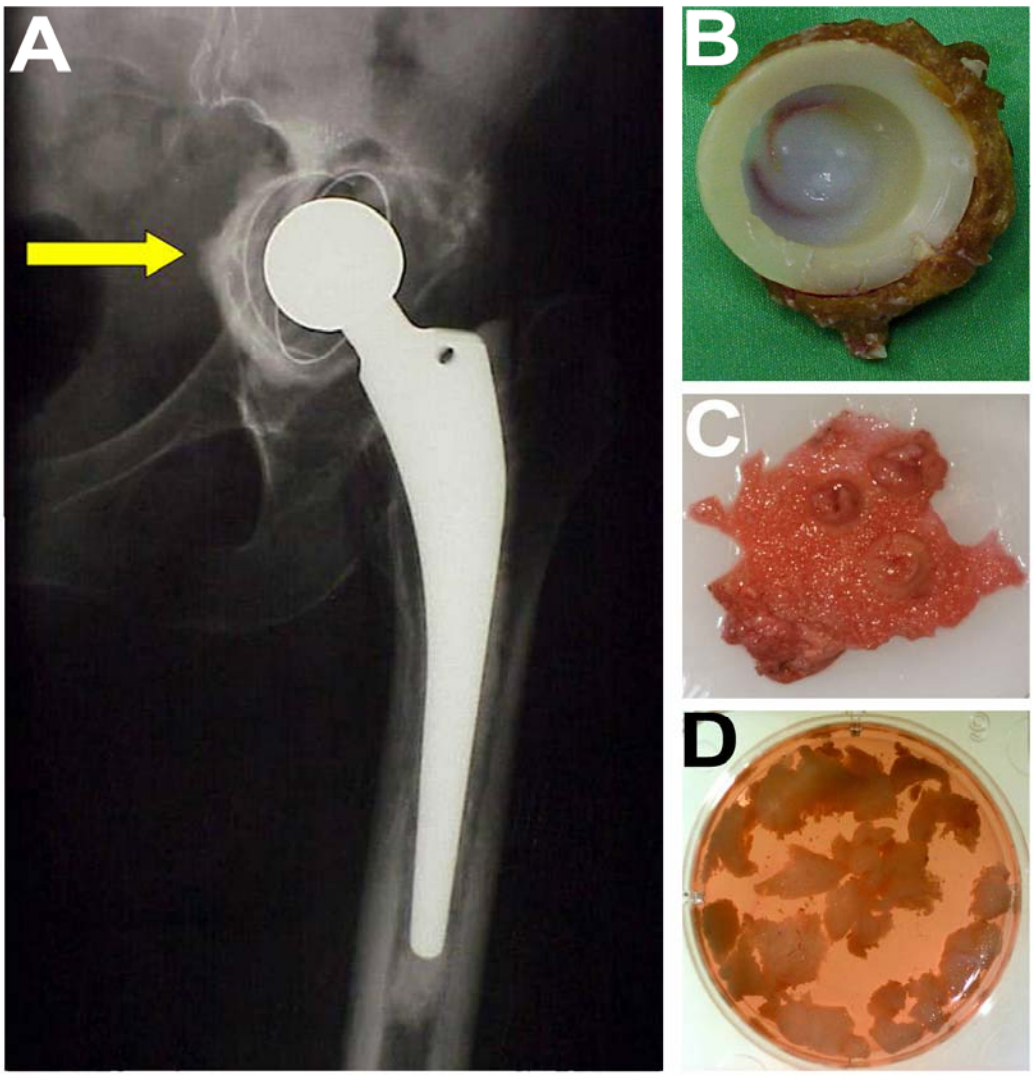

Figure 1. A, Loosened total hip implant. **Arrow** shows osteolysis around the cup. **B**, Removed cup surrounded by a pseudomembrane. **C**, Aggressive tissue of an interface membrane. **D**, Explant culture. Approximately 0.5g wet synovial or interface membrane tissue was cultured in 2.5 ml DMEM containing 5% FBS.

The interface membrane (IFM) is a granulomatous tissue consisting predominantly of fibroblasts, macrophages, and foreign body giant cells (**Fig. 2**) (1-4).

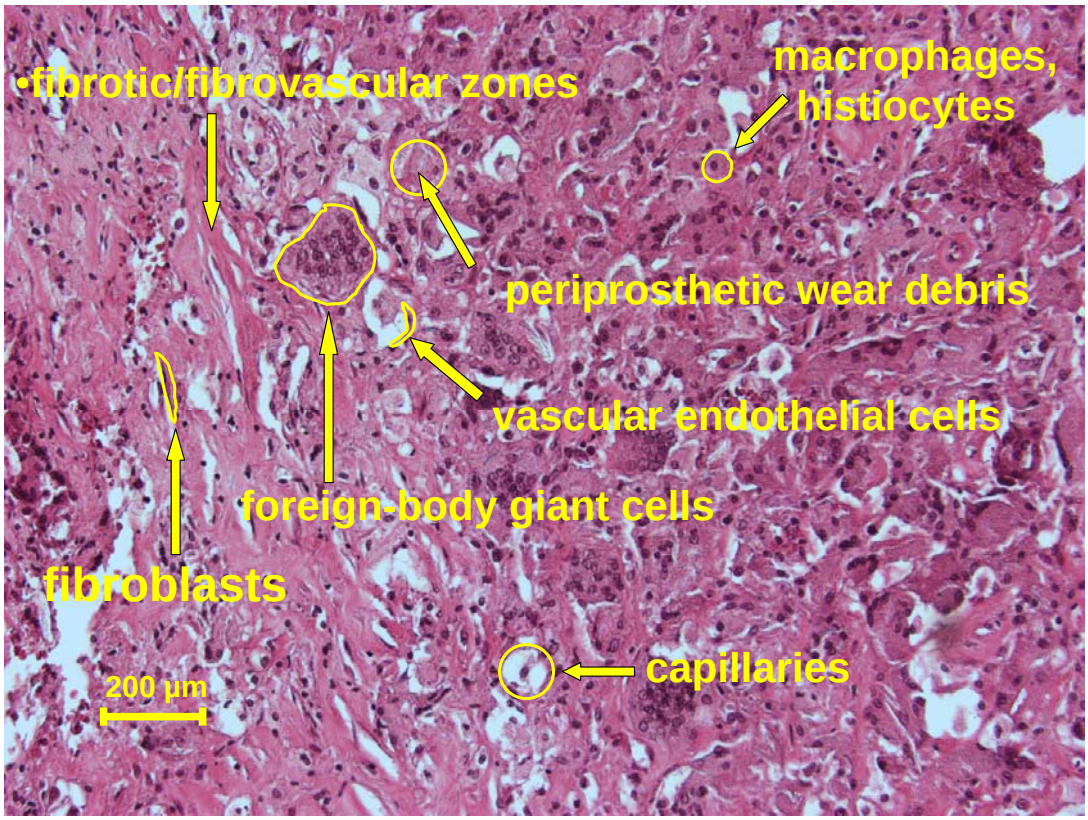

Figure 2. Interface membrane (Hematoxylin and Eosin staining).

It is believed that particulate wear debris via phagocytosis activates cells which then proliferate and produce inflammatory mediators, such as TNF-α, IL-1β and IL-6. These "bone-resorbing" agents activate eventually all cell types in the IFM in either a paracrine or autocrine manner (**Fig. 3**) (3,5,6).

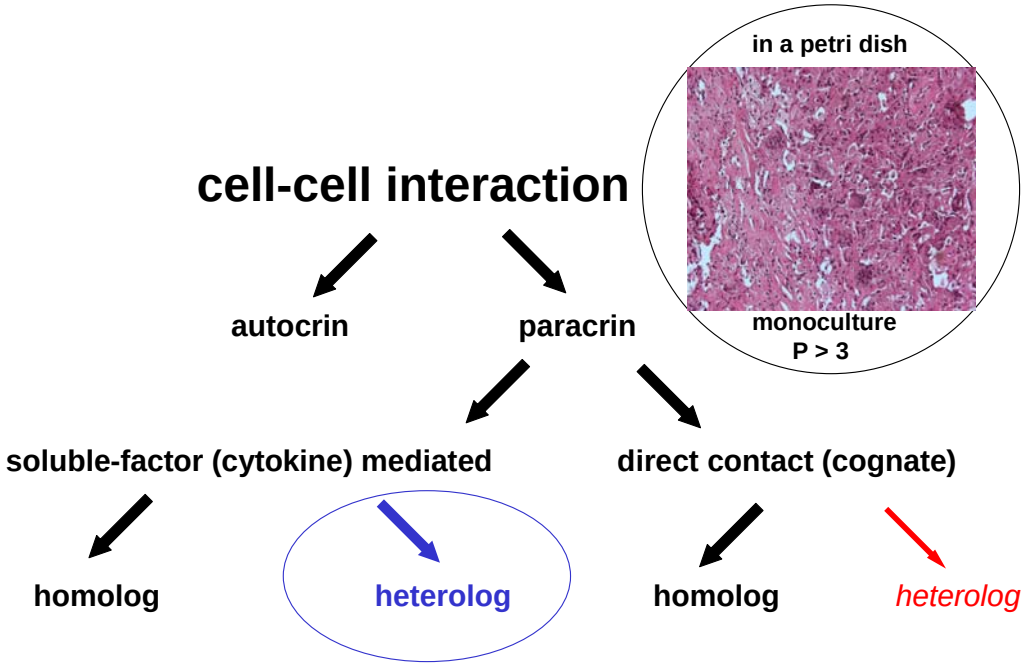

Figure 3. Cell-cell interactions *in vivo*. Black scripts represent previous *in vitro* models. Black+blue scripts represent our improved *in vitro* model. Black+blue+red scripts would represent a perfect model.

A large number of studies have shown that macrophages are activated by phagocytosed particles and produce inflammatory cytokines, which ultimately leads to osteoclastogenesis and increased bone resorption (4,7,8).

Osteoblasts also phagocytose particles, and particulate phagocytosis significantly increases the secretion of TNF-α, IL-6, and the cell surface expression of RANKL (9,10), simultaneously suppressing procollagen E1[I] transcription (11) via the activation of NF-KB signaling (12,13).

Information on the fibroblast (dominant cell type in the IFM) response to particle debris is less extensive (16-22). Thus, it still remains to be determined what role fibroblasts play in the pathogenesis of periprosthetic osteolysis associated with particle wear debris.

Several early studies dealt with the biological response to particulate debris.

Jiranek et al. used two techniques that go beyond the scope of traditional histology to demonstrate directly the presence of specific cytokines that have been implicated in pathological resorption of bone and to localize these products to individual cell types. These authors used the techniques of *in situ* hybridization and immunohistochemistry. *In situ* hybridization permits identification of the individual cells expressing increased steady-state levels of mRNA for a given product. It has the advantage of being able to demonstrate the relationship of these cells to a known stimulus. Immunohistochemistry allows the differential identification of cell types that may have similar histological appearances.

Jiranek et al. studied the interface membrane around total hip prostheses associated with evidence of aseptic loosening. Although *in situ* hybridization cannot be used to measure the actual release of cytokines, elevated steady-state levels of mRNA provide indirect evidence that these cytokines are being produced by certain cells.

The association of these cytokines with cells expressing monocyte-macrophage phenotype provides direct evidence that these cells have an important role in the recognition of foreign material and the release of inflammatory mediators. In the model of Jiranek et al., these mediators modulated the activity of neighboring cells (including bone cells) that are responsible for mediating the focal osteolysis (14).

Horowitz et al. demonstrated that phagocytosis of polymethylmethacrylate particles stimulates tumor necrosis factor, a cytokine that has been implicated in pathological resorption of bone. Horowitz et al. also established the importance of particle size in the determination of the pattern of the response. In addition, they demonstrated that the phagocytosis of cement is toxic to macrophages and results in cell lysis and death. They speculated that the release of mediators from dying cells may contribute to the capacity of cement to induce an inflammatory response. After cell lysis, the particulate debris may be released and thus re-enter the inflammatory cycle (15).

Maloney et al., described the *in vitro* fibroblastic response to specific metallic debris. This work is important because it demonstrated the capacity of so-called facultative phagocytes to respond directly to metal wear particles by undergoing cell proliferation. This response may contribute to the development of the fibrous tissue reaction observed in peri-implant tissues. Relatively low concentrations of titanium, titanium-aluminum alloy, and chromium stimulated cell proliferation markedly, as

measured by the uptake of radioactive thymidine. The observation that cobalt was toxic at all concentrations and that it resulted in a decreased uptake of thymidine is consistent with the previously documented toxicity of this metal. At high concentrations, all of these particles were toxic. Maloney et al. speculated that metallic debris may stimulate the formation of the fibrous membrane around implants that were inserted without cement. Furthermore, they cited the hypothesis that this membrane may act as a conduit for the polyethylene debris that was generated at the articular surface to enter the space between the implant and the bone. They hypothesized that such polyethylene may contribute to a potential synergistic interaction between metallic and polymeric wear debris, which potentiates the production of factors that enhance osteolysis and accelerate loosening of total joint implants (16).

Haynes et al. investigated the cellular responses to different metal-alloy wear particles of similar size on the release of the inflammatory mediators that have been implicated in bone resorption. The study was performed with macrophages, the cells that are probably the principal target for particle-mediated cell activation.

These results complement the investigations of Maloney et al. and of Horowitz et al., and they demonstrate the specificity of the response in different cell types. Haynes et al. found that particles of cobalt-chromium were toxic to cells. Particles of titanium alloy resulted in minimum cell toxicity and were more likely to cause the release of inflammatory mediators that have been implicated in osteolysis, indicating that the pro-inflammatory effect of particles (the capacity to elicit one or more events involved in inflammation) may be as harmful as, or even more harmful than, the toxic effect. Thus, if a less toxic particle stimulates continuing synthesis and release of inflammatory mediators, it may, for example, have a marked biological effect in terms of the capacity to induce bone resorption.

The high rate of failure of revision arthroplasties done without cement is believed, in part, to be related to the deficient bone stock that did not provide an adequate environment for the primary fixation with cement. Consequently, the potential for obtaining bone ingrowth with revision arthroplasties done without cement is less than that with primary arthroplasties done without cement (17).

Turner et al. noted the importance of an environment with suitable osteogenic potential if fixation is to be obtained by bone ingrowth. They investigated various models, including autogenous bone graft and bone substitutes such as hydroxyapatite

β-tricalcium phosphate, with the goal of maximizing the osteogenic potential at the site of implantation (18).

Horowitz et al., Maloney et al., and Haynes et al. used *in vitro* models based on cell culture to begin to define the factors that determine the outcome of metal and polymeric particle-cell interactions. These results extended the investigations in related fields and supplement the findings from earlier studies of materials used for orthopaedic implants. These previous investigations have helped to define the sequence of events associated with recognition and internalization of particles, although there are still many questions concerning the actual cellular and molecular processes by which these materials influence cell function.

Yao et al. found that fibroblasts isolated from IFMs of patients with failed total joint replacements, when challenged with small-sized (<3 μm) titanium (Ti) particles, responded with enhanced expressions of collagenase, stromelysin and, to a lesser extent, tissue inhibitor of metalloproteinases (19). These responses were similar to that measured in fibroblast cultures of rheumatoid synovium, but more pronounced than in normal synovial fibroblasts in the same experimental condition.

Moreover, particulate wear debris can directly activate fibroblasts to synthesize bone-resorbing metalloproteinases and possibly compounds which then suppress osteoblast functions critical for bone remodeling (11).

Some of the fibroblast-derived factors may have an autocrine effect, or may alter the responsiveness of other cells (macrophages, osteoclasts) in the periprosthetic environment (22-24). Reciprocally, different cell types of IFM can phagocytose particles, produce various cytokines, chemokines, and growth factors in response to particulate stimulation, which then modify the fibroblast function **(Fig. 3)**.

The conclusions drawn from these *in vitro* studies must ultimately be tested against observations gleaned from appropriate *in vivo* experiments and careful correlations and analyses of retrieved clinical specimens.

To mimic the *in vivo* conditions of periprosthetic pathologic bone resorption as closely as possible, we stimulated interface membrane fibroblasts with titanium particles **(Fig. 4)** and/or conditioned media from interface membranes. In order to reproduce *in vivo* conditions, particles of approximately the same size distribution as the wear debris present in periprosthetic tissue (6,43–45) were used to stimulate fibroblasts.

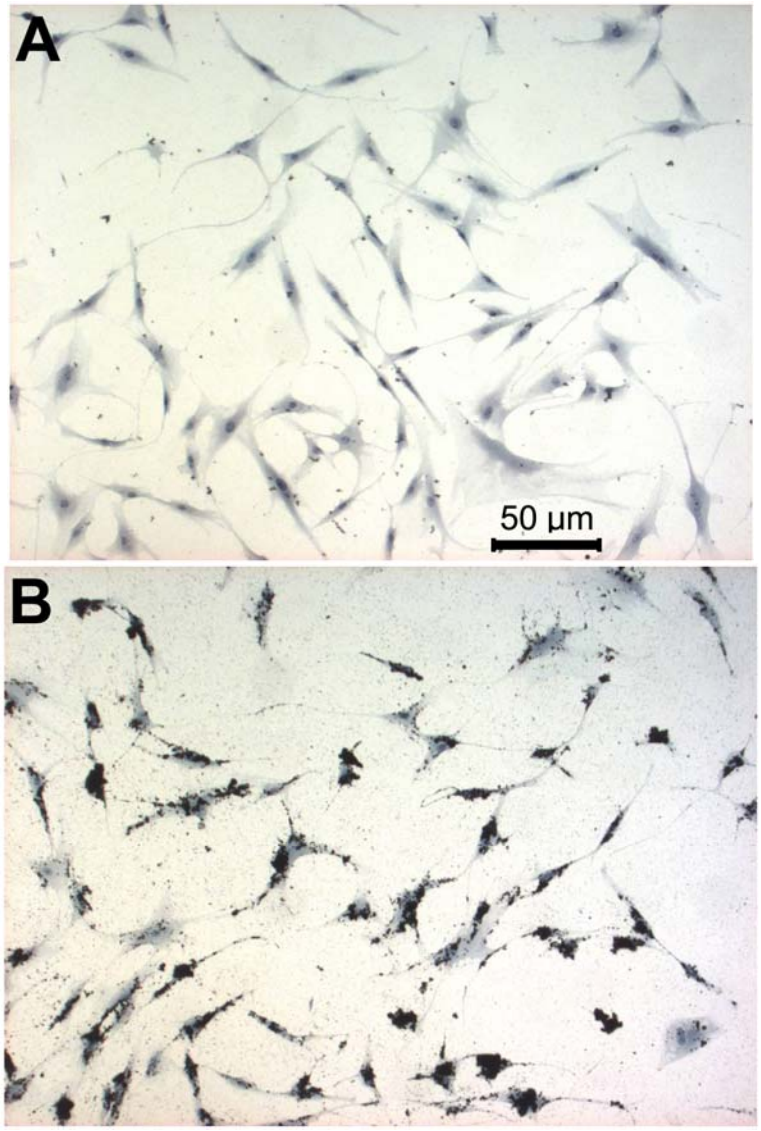

Figure 4. A, Untreated fibroblast culture (after 5 passages) isolated from an interface membrane. **B**, Same monoculture exposed to titanium particles overnight (0.075% [v/v]), and nonphagocytosed particles were washed out (light microscopy).

In this study we focused on the fibroblast response measuring the expression of MCP-1, IL-6 (fibroblast activation markers), IL-1β, VEGF, RANKL and OPG in response to stimulation with Ti particles and/or the conditioned media of IFM of loosened TJAs.

THEORY

Objectives

The aim of this study was to investigate how the fibroblasts respond to stimulation with particulate wear debris and/or conditioned media (CM) obtained from pathologic tissues, and whether these activated fibroblasts express compounds which are involved in bone resorption.

Methods

Titanium particles (a "prototype" wear debris), conditioned media (CM) from explant cultures of synovial tissues, periprosthetic soft tissues (interface membranes; IFMs) and proinflammatory cytokines were used to stimulate fibroblasts. RNase protection assay (RPA) was used to measure altered gene expression, and enzymelinked immunosorbent assay, Western blot hybridization and flow cytometry to determine protein expressions by fibroblasts. Tartarate-resistant acid phosphatase staining was used to identify multinucleated osteoclast-like cells.

MATERIALS AND METHODS

Particles and chemicals

All chemicals, unless otherwise indicated, were purchased from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Chicago, IL). Commercially pure small-sized titanium (Ti) particles (<3 μm; Johnson Matthey, Danvers, MA) were sequentially treated with 25% nitric acid overnight at room temperature and 0.1*M* sodium hydroxide in 95% ethanol at 30^{O}C for 10-12 hours three times (25), and then exhaustively washed with sterile, endotoxin-free phosphate buffered salt solution (PBS; pH 7.2). Endotoxin/lipopolysaccharide contamination of particles was excluded by using the high-sensitivity *Limulus* amoebocyte cell lysate assay (Biowhittaker, Walkersville, MD). Particles were autoclaved, sonicated and then sedimented in 10% human AB serum to remove a relatively larger size population of particles. The mean ± SD particle size was 1.42 ± 0.83 μm: 91% of these particles were smaller than 3 μm, and 72% smaller than 1 μm. Based upon the size distribution of these Ti particles (11,20), a 0.075% (volume/volume: v/v, used in this study) Ti suspension contained approximately 6 x 10^7 particles/ml. Ti particles were stored in Dulbecco's modified minimal essential medium (DMEM) at 4^{O}C.

Patients and tissue samples

The collection of human samples from joint replacement and revision surgeries, and the use of bone marrow aspirates, were approved by the Institutional Review Board; and samples were collected in consent with the patient. Although the major focus of this study was the interface membrane fibroblast (IFM-Fb) response to bioactive compounds released by cells of the interface membrane, synovial samples from normal joints (negative control) and rheumatoid joints (positive control) were also collected and tested in preliminary and comparative experiments. Normal synovial tissue samples were collected from the knee and ankle joints of 5 organ donors (age range 28-62 years) within 3-6 hr after death due to cardiovascular insufficiency or traffic accident. Additional "normal" synovial tissue samples were obtained from patients with femoral neck fractures with no evidence of synovial reaction and/or cartilage damage on histologic or radiographic analysis. Finally, a total of 12 normal synovial tissue samples were used for gene expression and cytokine assays. Synovial tissue from the knee joints of 8 patients with rheumatoid

arthritis (RA) (mean age 60.2 years, range 47–63 years) who underwent primary TJA surgery was collected. Periprosthetic interface membranes from loosened joint replacements (23 hip and 9 knee replacements) with osteolysis were obtained from patients during revision surgery, which took place an average of 10.1 years after the primary TJA. This group of patients consisted of 18 men and 14 women, with a mean age of 62.2 years (range 34–91 years). In addition to focal or diffuse osteolysis, the major reasons for revision surgery were pain, limited range of motion, and instability. The types of prosthesis and surgical procedure varied, as did the source of the tissue (from revision surgeries of hip or knee TJAs) and the original diagnosis that led to joint abnormalities and TJA (RA or osteoarthritis [OA]).

Explant cultures and conditioned media (CM)

Tissue samples in sterile containers of DMEM and 150 µg/ml gentamicin were transported from the operating room to the laboratory within 5-20 min after removal. Samples were minced (2-4 mm^3 in volume) in serum-free DMEM, washed, and representative tissue samples were distributed for explant cultures, RNA and fibroblast isolation, and histologic examination (**Fig. 1D**).

Approximately 0.5g wet synovial or interface membrane tissue was cultured in 2.5 ml DMEM containing 5% endotoxin-free fetal bovine serum (FBS, HyClone, Logan, UT), antibiotic/antimycotic solution, which was supplemented with 50 µg/ml gentamicin. Tissue samples were distributed in 12-well plates, and 90% of the medium was replaced daily for a total of seven days (26,27). Media which were harvested every 24 hours were centrifuged at 2500*g* for 10 min, and aliquots were reserved for cytokine assays, and stored at –20^{O}C until the explant culture system was completed. DMEM containing 5% FBS without tissue samples (medium control) was also incubated for 24 hours at 37^{O}C, harvested, centrifuged, and stored in the same manner as all other conditioned media.

Fibroblast isolation

Fibroblasts were isolated from both fresh tissues and 7-day-old explant cultures of synovial tissues to compare the yield and viability of fibroblasts from the corresponding tissue samples. Fibroblasts were isolated by pronase and collagenase digestions as described (19,20). Dissociated cells were washed with PBS and plated in Ø10cm petri dishes (Beckton Dickinson, Franklin Lakes, NJ) in DMEM/10%

FBS. Non-adherent cells were discarded the next morning by washing, and adhered cells (mostly fibroblasts) were cultured in DMEM/10% FBS. The yield of fibroblasts varied from sample to sample, but approximately the same number of viable cells (85-95%, determined by trypan blue exclusion test) were isolated from the fresh tissue and 7-day-old explant cultures.

Medium from fibroblast cultures was changed twice a week. Confluent monolayer fibroblast cultures were passaged at least five times and then passaged at ~0.7 x 10^6 cell density per Ø10cm petri dish for experiments. The fibroblast phenotype of isolated cells was confirmed by flow cytometry analysis (FACSCalibur, CellQuest software program, Beckton Dickinson) using anti-CD90 (Thy-1) (28) monoclonal antibody (mAb) (BD Pharmingen/ Bioscience, San Diego, CA) and by immunohistochemistry in 8-well chamber slides (Nalgene) using fluorochrome-labeled mAb 5B5 to F-subunit of propyl-4-hydroxylase (Dako Corporation, Carpenteria, CA). Alexa Fluor® 546 phalloidin (Molecular Probes, Eugene, OR), which binds to F-actin filaments was used to contour the intracellular localization of phagocytosed particles.

Freshly isolated fibroblasts from interface membranes contained particles (**Fig. 5A**), whereas the number of particles diminished during subsequent passages. After three-four passages the presence of particles was rare, and we could not detect macrophages or cells of the monocyte/macrophage lineage (CD11b+ cells)(BD Pharmingen) with flow cytometry; 99-100% of the cells were CD90+ fibroblasts. To mimic the *in vivo* condition and to determine whether fibroblasts could indeed phagocytose particulate wear debris *in vitro*, fibroblasts isolated from the IFM were treated with Ti particles (0.075%, v/v) and passaged into a chamber slide the next day. As shown in **Figure 4B** and **5B**, *in vitro* cultured fibroblasts phagocytosed a substantial number of Ti particles.

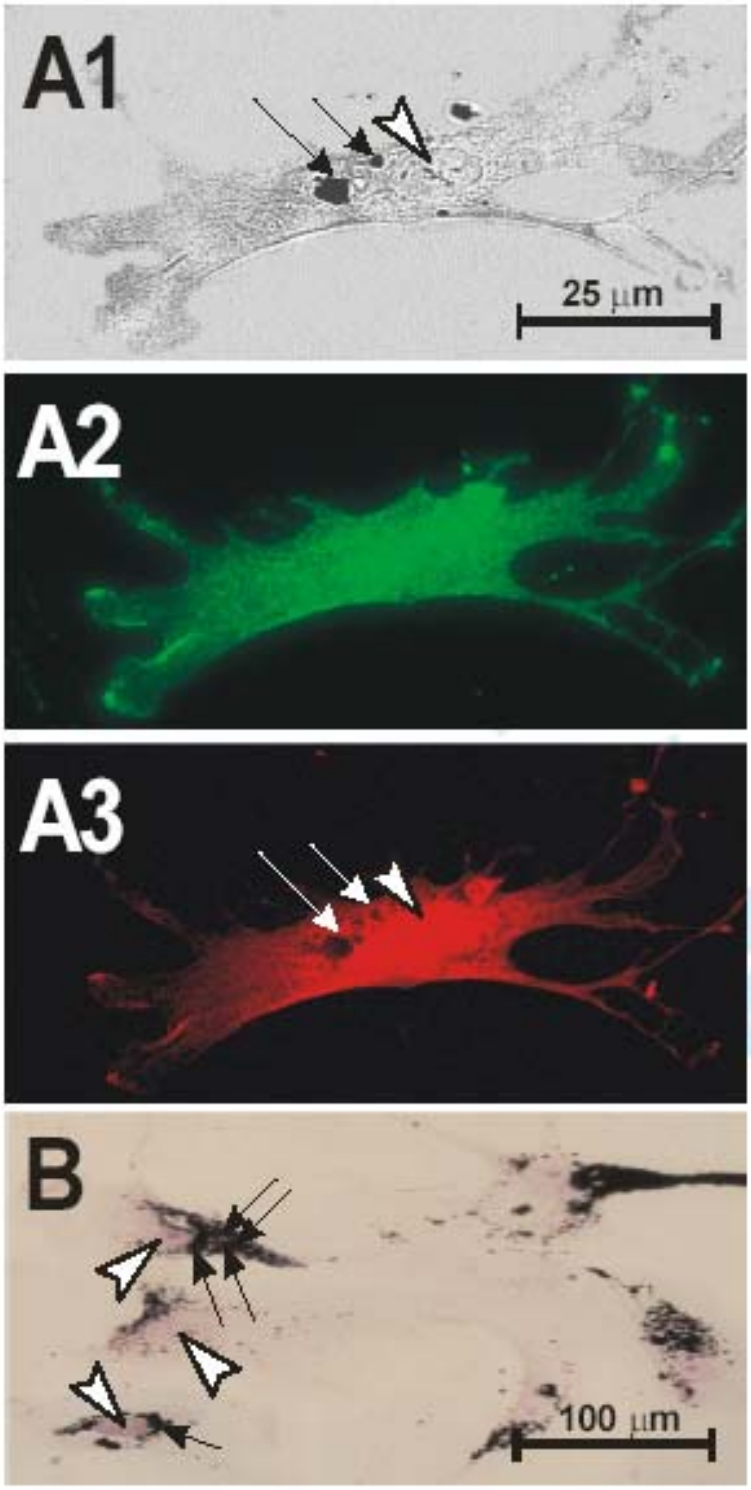

Figure 5. Interface membrane fibroblasts with phagocytosed particles
A, Images of the same freshly isolated fibroblast from an interface membrane. **A1**, Phase-contrast image. **A2** and **A3**, Fibroblast cultures double-stained with fluorescein isothiocyanate (FITC)–labeled monoclonal antibody to fibroblastspecific CD90 (green for surface staining) (**A2**) and Alexa Fluor 546 phalloidin (red for actin) (**A3**). Confocal z-series were projected into a single image to demonstrate the intracellular localization of 1 large and a few smaller particles in the interface membrane fibroblast. **Arrows** show metal particles; **arrowheads** show nuclei.
B, Fibroblast culture (after 5 passages) exposed to titanium particles overnight (0.075% [v/v]). Nonphagocytosed particles were washed out, and cells were stained with crystal violet for light microscopy. **Arrowheads** show nuclei; **arrows** show phagocytosed particles.

Treatment of fibroblasts with CM, Ti particles, or both

Confluent fibroblast cultures were subjected to 5% FBS containing DMEM for 24 hr, and then this medium was replaced with either DMEM with 5% FBS pre-incubated at 37^{O}C in CO_2 incubator (medium control) or conditioned media (without dilution) with or without titanium particles for different time periods. We simultaneously tested fibroblasts derived from 2 normal, 2 rheumatoid, and 3 osteoarthritic synovial tissue samples, and data in this report summarize the responses of 2–4 independent fibroblast lines isolated from interface membranes. In preliminary experiments, we determined the minimum and maximum concentrations of conditioned media harvested from interface membrane explant cultures, and the "optimal" concentration of titanium particles. Undiluted conditioned media and 0.075% (v/v) titanium particle concentrations were selected to achieve an average, usually the maximum, dose-dependent effect on fibroblast stimulation determined in the present study and previous experiments (19). Culture media were collected from all particle-stimulated and non-stimulated fibroblast cultures at various time points (from 6 hours to 96 hours).

Finally, we selected 16 conditioned media from the 32 explant cultures of interface membranes (CM-IFMs). The presence of particle wear debris in the the interface membrane tissues was retrospectively confirmed histologically. Prior to the experiments on fibroblast stimulation, conditioned media from interface membranes were individually pretested for 48 hours for cytokine production in cultures using the same normal synovial fibroblast cell line. The major selection criteria were that these conditioned media from interface membranes contained the highest and comparable amounts of proinflammatory cytokines and chemokines, and induced approximately the same amounts of IL-6, VEGF and IL-1β. Based on the response of this normal synovial fibroblast cell line (cytokine production), 4 groups were formed, each containing 2–5 conditioned media from interface membranes. These conditioned media from interface membranes were pooled and filtered through a 0.22 μm polycarbonate filter (Costar, Cambridge, MA), divided into aliquots, stored at –80^{O}C. Each of these four pools were then tested at least 2, usually 3-4, independent interface membrane fibroblast cell lines (with and without titanium particles), and the results are summarized in this study.

To investigate whether the source of donor tissue (the interface membrane) may affect fibroblast response, each pool of the conditioned media was prepared so

that the interface membranes were derived from patients who underwent TJA either due to rheumatoid arthritis (1 group) or osteoarthritis (3 groups), and who had the primary TJA 6–9 years prior to the revision surgery.

The other 16 conditioned media from interface membranes either did not fulfill the selection criteria, or the volumes were insufficient for complete experiments.

We have also used fibroblast cell lines derived from normal (n=2) and rheumatoid (n=2) synovium. The responses of these cells to either conditioned media or titanium, or both, were comparable with those found in interface membrane fibroblast cultures after 5–6 passages of cells (results not shown).

***In vitro* osteoclastogenesis assay**

Bone marrow aspirates were obtained from either iliac crest or vertebral bodies from men and women (age range 28–57 years) undergoing spine fusion procedures. Culture conditions, isolation and characterization of cells were the same as previously described (13,29), except that these bone marrow-derived progenitor cells were used for osteoclast formation. Briefly, buffy coat-separated nucleated bone marrow cells (2×10^7 per T75 tissue culture flasks) (Corning Inc., Corning, NY) were cultured in alpha-minimal essential medium (GIBCO Invitrogen Corporation, Carlsbad, CA) containing 10% FBS for 12-24 hours, and then adherent cells were gently trypsinized and transferred to 4-well chamber slides (Nalgene Nunc International, Naperville, IL) precoated with semiconfluent layer of interface membrane fibroblasts that had been left untreated, or pretreated with titanium, TNF-α or conditioned media from interface membranes.

Osteoclastogenesis in these cocultures was induced by adding 50 ng/ml recombinant macrophage colony stimulating factor (M-CSF; R&D Systems Minneapolis, MN) for 8-14 days. Fibroblasts were stained for CD90 and RANKL, fluorescent images were stored, and then restained for tartarate-resistant acid phosphatase (TRAP, Sigma). Multinucleated (> 4 nuclei per cell) TRAP+ cells were counted. Unstimulated interface membrane fibroblasts, interface membrane fibroblasts stimulated with M-CSF alone, interface membrane fibroblasts stimulated with RANKL alone, or bone marrow cells treated with M-CSF alone were used as negative controls. Bone marrow cells treated with M-CSF (50 ng/ml) plus RANKL (100 ng/ml; R&D Systems) were used as positive controls. Additionally, fibroblasts

were plated (5 x 10^4 cells/well in a 24-well plate) and pretreated with titanium particles (0.075% [v/v]) for 48 hours, and then particulate wear debris was removed by exhaustive washing. Subsequently, fresh bone marrow cells (0.8 x 10^6/well) and 50 ng/ml M-CSF were added to multiple wells. Fibroblasts alone, bone marrow cells alone, fibroblasts and bone marrow cells together, with or without M-CSF, or untreated fibroblasts (as described above) were used as negative control cultures.

RNA isolation and RNase protection assay (RPA)

Fresh tissue samples (~0.2-0.4g), and tissue samples cultured for 7 days, were homogenized on ice with a polytron homogenizer (KRI Works, Cincinnati, OH). Homogenate was centrifuged to remove large debris, and RNA was extracted with TRIzol (Invitrogen) as previously described (13,19). TRIzol was also used to isolate total RNA from cultured fibroblasts before and after treatments with titanium, conditioned media, or titanium plus conditioned media at 6, 12, 24, 48, and 72 hours. The amount of RNA was determined using a RiboGreen RNA quantification kit (Molecular Probes) (13).

RPA was performed on 8 μg of RNA using the Riboquant Multiprobe RNase Protection Assay System, according to the recommendations of the manufacturer (BD PharMingen). In addition to commercially available cytokine, chemokine, and growth factor templates (BD Biosciences) mostly used in preliminary studies and for the selection of cytokines and chemokines, 2 custom-made RPA templates were purchased from BD PharMingen/Biosciences. The no. 65184 custom-made template was designed to quantify the expression levels of human TNF-α, IL-1 receptor type I, IL-4, matrix metalloproteinase-1, IL-1α, IL-1β, MCP-1, transforming growth factor-β1 (TGF-β1), TGFβ receptor type I, and interferon-γ; and template no. 65120 was designed to determine a set of angiogenic factors, such as RANTES, interferon-γ-inducible 10-kD protein, cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2), basic fibroblast growth factor, fibroblast growth factor receptor, IL-8, angiopoietin 1, VEGF, and c-myc. Custommade templates included housekeeping genes L32 and glyceraldehyde-6-phosphate dehydrogenase (GAPDH).

Single-stranded labeled ([E-32P]UTP) antisense RNA probes were synthesized by *in vitro* transcription from a complementary DNA (cDNA) template provided in the Riboquant Multiprobe RNase Protection Assay kit. Antisense RNA probe was purified by phenol–chloroform extraction and ethanol precipitation and

was hybridized with messenger RNA (mRNA) samples overnight at 56^{O}C. RNase A and RNase T were used to digest nonhybridized single-stranded RNA. Protected double-stranded RNA was purified by phenol–chloroform extraction followed by ethanol precipitation, and then the 32P-labeled samples of different lengths were separated on 5% denaturing polyacrylamide/8M urea gel, as previously described (30). Radioactivity of the samples was measured and analyzed by scanning densitometry on a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA). I found a high correlation (> 95%) between the amount of ribosomal RNA and the message levels of the housekeeping genes L32 and GAPDH. However, L32 expression was more consistent in titanium-stimulated fibroblast cultures, and all samples were normalized to L32.

Reverse transcription real-time quantitative polymerase chain reaction (QRT-PCR)

Since neither RANKL nor OPG probe for RPA template was available at the time of these experiments, the mRNA levels of these compounds were determined by real-time quantitative PCR using the Smart Cycler System (Cepheid, Sunnyvale, CA). The detection was carried out by measuring the binding of fluorescent SYBR Green-I to double stranded DNA. The PCR reactions were carried out in microtubes in 25 µl volume. Real-time quantitative PCR efficiencies for all probes were confirmed using three different template dilutions in all of the runs. The cDNA template in 1 µl reverse transcriptase PCR product (equivalent with ~0.025-0.1 µg total RNA) was added to a PCR mixture which contained final concentrations of 0.5 µ*M* RANKL-specific forward primer (5'-CGT TGG ATC ACA GCA CAT CAG) and reverse primer (5'-GCT CCT CTT GGC CAG ATC TAA C) or OPG-specific forward primer (5'-GCA GCG GCA CAT TGG AC) and reverse primer (5'-CCC GGT AAG CTT TCC ATC AA), a 1:50,000 dilution of SYBR Green-I stock solution (BioWhitaker Mol. Appl. Cambrex, Rockland, ME), 200 µ*M* dNTP, 1.5 m*M* $MgCl_2$, and 2.5 units of *Taq* DNA polymerase (Promega, Madison, WI). For normalization, L32 cDNA was amplified with forward primer (5'-CAA CAT TGG TTA TGG AAG CAA CA) and reverse primer (5'-TGA CGT TGT GGA CCA GGA ACT).

The fluorescence emitted by the reporter dye was detected online in real-time, and the threshold cycle (Ct) of each sample was recorded as a quantitative measure

of the amount of PCR product in the sample as previously described (31). The RANKL and OPG signals were normalized against the quantity of L32 and expressed as $\Delta C_t = C_{tRANKL} - C_{tL32}$). The differences in RANKL signals were expressed as $\Delta\Delta C_t = \Delta C_{tTreated} - \Delta C_{tUntreated}$ in all different treatment groups. Relative gene expressions were than calculated as $2^{-\Delta\Delta Ct}$.

The real-time PCR assays were repeated 3 times using 3 independent reverse-transcribed RNA samples isolated from 3 untreated fibroblast cultures and the 3 corresponding fibroblast cultures treated with either titanium particles, conditioned media from interface membranes, or both, at each time point. The same RNA samples that were used for reverse transcription were also used for RPA.

Detection of specific protein products by enzyme-linked immunosorbent assay (ELISA)

All conditioned media harvested from explant cultures of synovial tissues and interface membranes, and from treated and untreated fibroblasts after 6–96 hours, were analyzed by ELISA. Conditioned media were harvested, centrifuged, and aliquots were stored at –70^{O}C. Levels of TNF-α, IL-1β, MCP-1, IL-6, IL-8, and VEGF were determined using capture ELISAs (R&D Systems Minneapolis, MN).

Detection of RANKL by Western blot hybridization and flow cytometry

To detect soluble forms of RANKL, the most potent osteoclastogenic and activation factor produced by fibroblasts treated with titanium or conditioned media from interface membranes, the harvested tissue culture media were loaded on sodium duodecyl sulfate (SDS)-10% polyacrylamide gels (PAGE) under reducing conditions. To detect nonsecreted (possibly membrane-bound) RANKL, treated and untreated cells were lysed in ice-cold lysis buffer (50 m*M* Tris HCl [pH 8.0], 150 m*M* NaCl, 0.1% SDS, and 1% Nonidet P-40) containing protease inhibitors (1 m*M* phenylmethylsulfonyl fluoride and 1 unit/ml aprotinin), phosphatase inhibitors (50 m*M* NaH_2PO_4, 10 m*M* Na-pyrophosphate, 50 m*M* KF, and 1 m*M* Na_3VO_4), and 0.1% NaN_3 for 1 hour at 4^{O}C.

Cell lysates were cleared by centrifugation, and 15 μg of protein per lane was separated by SDS–10% polyacrylamide gel electrophoresis under reducing conditions. Proteins were electrophoretically transferred onto nitrocellulose membranes (BioRad, Hercules CA), and membranes were blocked with 1% fat-free

milk, and stained with anti-RANKL mAb (clone 70513; R&D Systems) or rabbit polyclonal antibody (Santa Cruz Biotechnology, San Diego, CA). The 24 and 48 kDa bands were identified with recombinant human RANKL (Santa Cruz Biotechnology). Enhanced chemiluminesence (Amersham, Arlington Heights, IL) was used to detect immune reactions.

For flow cytometry, untreated or treated confluent fibroblast cultures were harvested with enzyme-free cell dissociating buffer (Gibco), and then washed 3 times in washing buffer (PBS containing 1% bovine serum albumin). Cells were resuspended in 100 µl washing buffer and incubated with 10 ng/µl of mouse anti-human-RANKL mAb (clone 70513) for 1 hour at $4^{O}C$, followed by biotin-labeled goat anti-mouse Ig antibody (10 ng/µl; BD Pharmingen). The reaction was developed with streptavidin-phicoerythrin (Gibco). Samples were fixed in 2% formalin and then analyzed by FACSCalibur using CellQuest software (Becton Dickinson, San Jose, CA). An IgG1 isotype control mAb was used to determine nonspecific background levels in all experiments.

Statistical analysis

Descriptive statistics were used to determine group means and standard error of the mean (SEM). The Pillai's trace criterion was used to detect multivariate significance. Subsequently, the Mann-Whitney U test was performed to compare the results of experimental groups. *P* values less than 0.05 were considered significant. All statistical analyses were performed with SPSS/PC+ version 10.1 (SPSS, Chicago, IL).

RESULTS

Selection of ”bone-resorbing” factors

In the first set of experiments, synovial tissue samples from normal and rheumatoid joints were analyzed and their gene expression levels and corresponding cytokine/chemokine secretions were compared to those measured in periprosthetic (interface membrane) soft tissues.

For this purpose, we used standard, commercially available Riboquant Multiprobe RPA templates (hCK3, hCK4, hCK26, hCR4, hCR5, hCR6 and hAngio-1). After prescreening up-regulated genes on different RPA templates, we selected the hCK4 template and designed 2 custom-made templates to measure altered gene expressions of interest (**Figure 6**).

Although a number of cytokines, chemokines, and angiogenic factors were measured by ELISA or detected by Western blot hybridization, only the ELISA results of TNF-α, MCP-1, IL-1β, IL-6, IL-8 and VEGF are shown (**Figure 7C**).

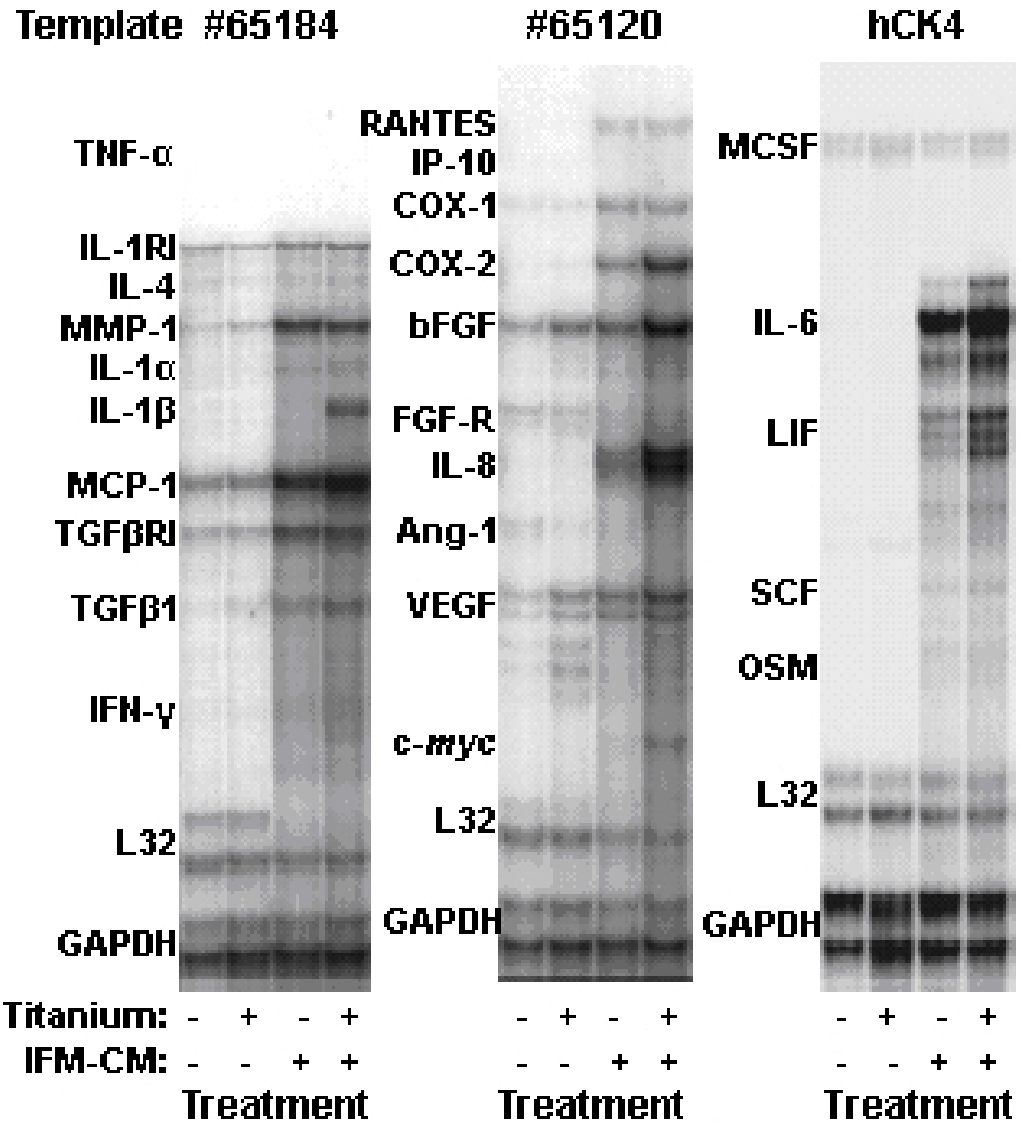

Figure 6. Effect of titanium (Ti) particles and conditioned media (CM) from interface membranes (CM-IFM) on gene expression by fibroblasts isolated from the interface membrane.
Results shown are from Rnase protection assays using radiolabeled complementary RNA probes from untreated interface membrane fibroblasts and interface membrane fibroblasts treated for 48 hours with titanium and/or conditioned media, on an hCK4 template and 2 custom-made templates. ^{32}P-labeled transcripts of known size were generated by *in vitro* transcription from RNA templates and were used as size markers. Dashes indicate the corresponding gene's position (occasionally 2 or 3 transcripts can be seen), whereas the intensities of bands show the level of mRNA expression.
Expression of the housekeeping gene L32 correlated (>95%) with 18S ribosomal RNA and was used for normalization in all experiments. GAPDH (glyceraldehyde-6-phosphate-dehydrogenase) showed more variability in response to treatments; thus, it was not used for normalization.
Representative panels from >15 hybridization experiments using 3–4 independent interface membrane fibroblast lines and 4 conditioned media from interface membranes are shown.
Abbreviations. **Template 65184:** TNF-α = tumor necrosis factor-α; IL-1α/β = interleukin-1α/β; IL-1RI = IL-1 receptor type I; MMP-1 = matrix metalloproteinase-1 (collagenase); MCP-1 = monocyte chemoattractant protein 1; TGF-β1 = transforming growth factor-β1; TGF-βRI = TGF-β receptor type I; IFN-γ = interferon-γ. **Template 65120:** RANTES: a CXC chemokine regulated upon activation normally T-cell expressed and secreted; IP-10 = CXCL-10 = CXC chemokine = interferon-γ-inducible 10-kDa protein; Cox-1/2 = cyclooxygenase 1 and 2; bFGF = basic fibroblast growth factor; FGF-R = fibroblast growth factor receptor; Ang-1 = angiopoietin-1; VEGF = vascular endothelial growth factor; c-*myc* = oncogene. **Template hCK4:** MCSF = macrophage colony-stimulatory factor; IL-6 = interleukin-6; LIF = leukemia inhibitory factor; SCF = stem cell factor; OSM = oncostatin M.

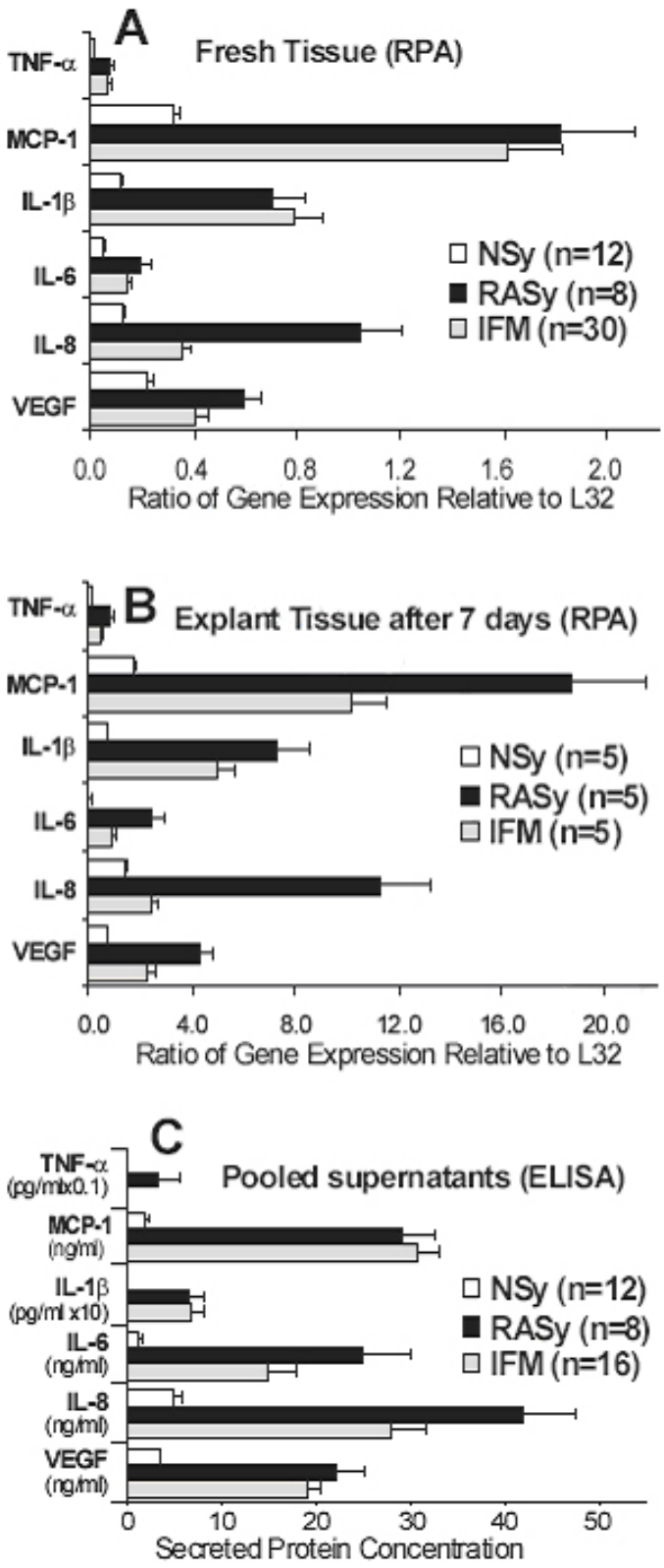

Figure 7. A and **B**, Steady-state mRNA levels measured in fresh normal synovial tissue (NSy), rheumatoid synovial tissue (RASy), and interface membranes (IFM), either freshly isolated **(A)** or after a 7-day culture period **(B)**. **C**, Secreted cytokine, chemokine, and VEGF levels measured in pooled conditioned media of explant cultures. Levels of mRNA were measured by RNase protection assay (RPA) normalized to the expression of the housekeeping gene L32 in the same sample, and cytokine levels were determined by capture enzyme-linked immunosorbent assay (ELISA). Values are the mean and SEM. See Figure 2 for other definitions.

Steady-state mRNA levels in interface membrane and synovial tissues, and secreted cytokines/chemokines in conditioned media

As summarized in **Figure 7** for selected compounds, normal synovial tissue expressed significantly lower message levels for all cytokines, chemokines, growth factors and angiogenic factors than did either synovial samples from rheumatoid joints or interface membrane tissue samples (**Figure 7A**). The gene-specific mRNA expression continuously increased until day 7, the final day of explant cultures (**Figure 7B**), when the message levels for all measured genes were approximately 4-10-fold higher than in fresh samples (**Figure 7A**). These findings corresponded to the results of measurement of secreted cytokines and growth factors (**Figure 7C**).

Selection of fibroblasts and fibroblast activation markers

Originally, we collected conditioned media from normal and rheumatoid synovium, and conditioned media from interface membranes (**Figure 7**), and tested fibroblasts of different origins. IL-1β, MCP-1 and IL-6 seemed to be the most sensitive and consistent markers of fibroblast activation. We found slight, but not significant, differences in fibroblast responses when the same conditioned media (with or without titanium particles) were tested on different fibroblast cell lines.

Finally, as described in above, 16 conditioned media from interface membranes were combined in 4 pools, and these pooled conditioned media were tested in 2-4 interface membrane fibroblast cell lines. In addition, we prepared an artificial "cytokine cocktail" (used as a positive control) based on the concentrations of 6 cytokines/chemokines measured in conditioned media from interface membranes (**Figure 7C**).

With this cocktail, we observed similar levels of IL-6, IL-1β, and MCP-1 secretion by interface membrane fibroblasts as were found when conditioned media from pathologic samples (either rheumatoid synovium or interface membrane) were used (**Figure 8**).

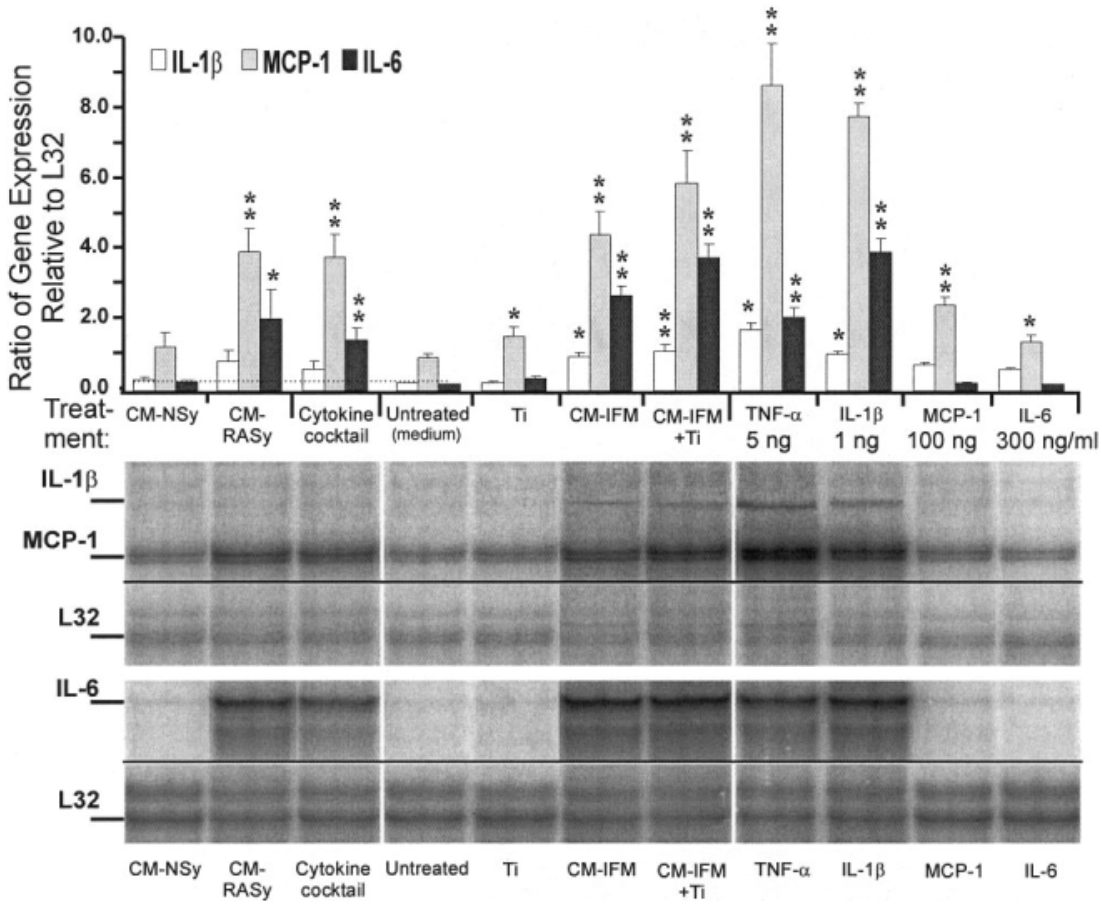

Figure 8. Expression of mRNA for IL-1β, MCP-1, and IL-6 in interface membrane fibroblasts cultured for 24 hours in normal Dulbecco's minimum essential medium with 5% fetal bovine serum, and in conditioned media harvested from explant cultures of normal synovial tissue (CM-NSy) and rheumatoid synovial tissue (CM-RASy), interface membranes from loosened implants (CM-IFM), and a "cytokine cocktail" containing 3 pg/ml TNF-α, 80 pg/ml IL-1β, 150 ng/ml IL-6, 300 ng/ml IL-8, 20 ng/ml VEGF, and 50 ng/ml MCP-1. This cocktail concentration was selected to represent approximately the same cytokine/chemokine and VEGF concentrations that were measured in pooled CM-IFM (Figure 3C). In general, the combination of titanium (Ti) and CM-IFM had a synergistic effect. TNF-α and IL-1β, when used alone, exerted the highest effect on MCP-1 and IL-6 expression after 48 hours of treatment. Findings with all treatments were normalized to L32 expression. Values are the mean and SEM of 9–15 RNase protection assays (RPAs) (48-hour treatments using 3–4 interface membrane fibroblast lines). $* = P < 0.05$; $** = P < 0.01$, versus untreated samples. Lower panels show strips of representative RPA membranes using the same interface membrane fibroblast line. See Figure 6 for other definitions.

Expression of mRNA and secretion of proteins by fibroblasts exposed to titanium particles, conditioned media from interface membranes, or both

We showed that fibroblasts could phagocytose particles either *in vivo* (**Figure 5A**), or *in vitro* (**Figure 4B** and **5B**), and that fibroblasts responded to stimulation with titanium particles, inflammatory cytokines, and conditioned media (**Figure 8**). The response was observed at both the transcriptional and the translational levels. Therefore, we were interested in (1) correlations between titanium-induced and conditioned media–induced gene expression, (2) the level and time frame of titanium-induced and conditioned media–induced gene expressio, and (3) which of the genes that code for the most relevant bone-resorbing agents are significantly affected by stimulation with either titanium particles or conditioned media. To investigate these gene characteristics, interface membrane fibroblasts were left untreated (i.e., cultured in medium control), or treated with conditioned media from interface membranes with or without 0.075% (v/v) titanium particles. Titanium particles had an effect, but relatively moderate one, on the expression of genes selected on the 3 RPA templates (**Figure 6**). Among the genes differentially expressed in the cultures treated with conditioned media from interface membranes versus the untreated cultures, MCP-1, IL-6, IL-8, TGFβ1, VEGF, Cox-1, and Cox-2 were the most prominently expressed.

These genes were expressed at even higher levels with combination treatment (conditioned media from interface membranes plus titanium).

Titanium particles and conditioned media from interface membranes exhibited either additive or synergistic effects on MCP-1, IL-8, Cox-2, IL-6 and leukemia inhibitory factor (LIF), all of which are known to be involved in osteoclast maturation and activation (**Figure 6**).

Interface membrane fibroblasts responded to treatment in both a dose-dependent manner (data not shown) and a time-dependent manner.

In general, with the 3 selected fibroblast activation markers (IL-1β, MCP-1 and IL-6) the highest gene expression was achieved between 12 and 48 hours of treatment, whereas the highest amounts of secreted proteins (except IL-1β) were measured in culture media between 72 and 96 hours (**Figure 9**).

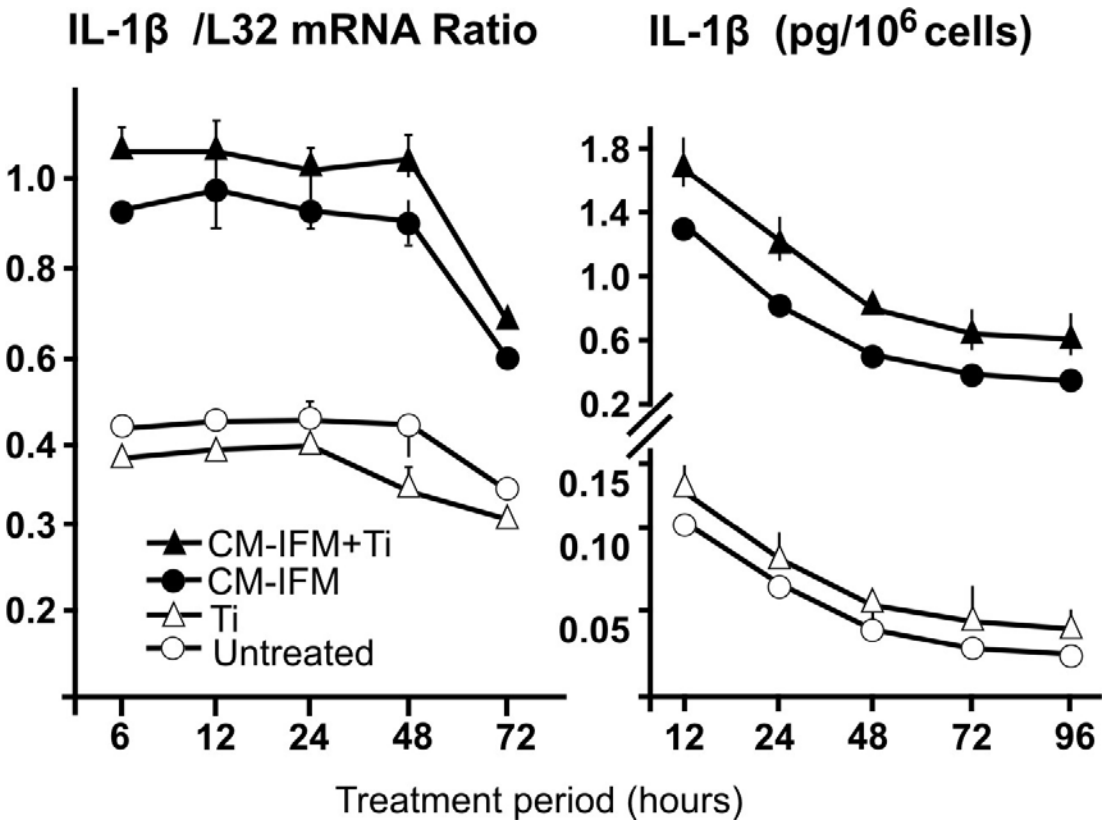

Figure 9A. Time-dependent responses of interface membrane fibroblasts to stimulation with conditioned media from interface membranes (CM-IFM) in the presence or absence of titanium (Ti) particles (0.075% [v/v]). Left panel summarizes the expression of mRNA for IL-1β, and right panel shows levels of the corresponding proteins secreted into the medium. IL-1β levels represent the amount of proteins actually secreted (their "original" concentrations in the CM-IFM [incubated without fibroblasts] have been subtracted). Note the picogram levels of IL-1β. Values are the mean ± SEM of triplicate wells from 3–5 independent experiments. See Figure 6 for other definitions.

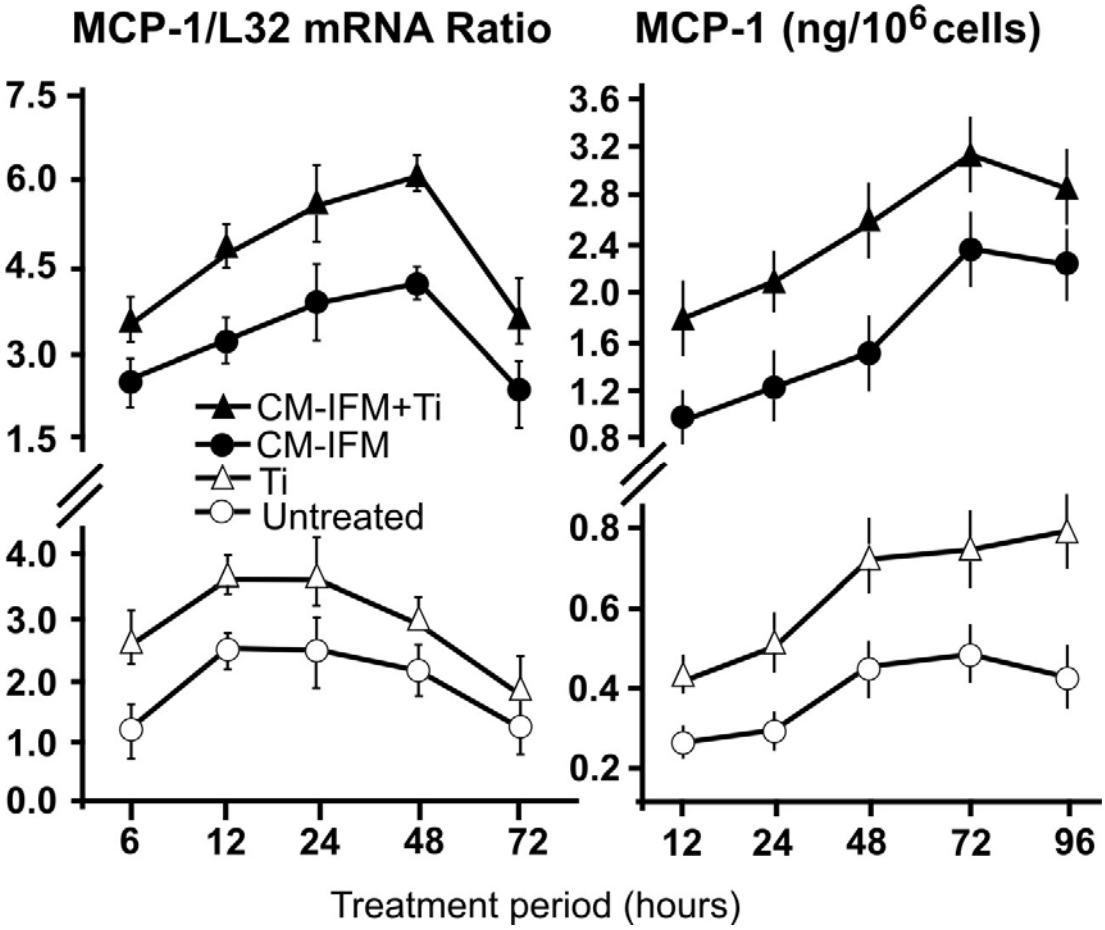

Figure 9B. Time-dependent responses of interface membrane fibroblasts to stimulation with conditioned media from interface membranes (CM-IFM) in the presence or absence of titanium (Ti) particles (0.075% [v/v]). Left panel summarizes the expression of mRNA for MCP-1, and right panel shows levels of the corresponding proteins secreted into the medium. MCP-1 levels represent the amount of proteins actually secreted (their "original" concentrations in the CM-IFM [incubated without fibroblasts] have been subtracted). Note the nanogram levels of MCP-1. Values are the mean ± SEM of triplicate wells from 3–5 independent experiments. See Figure 6 for other definitions.

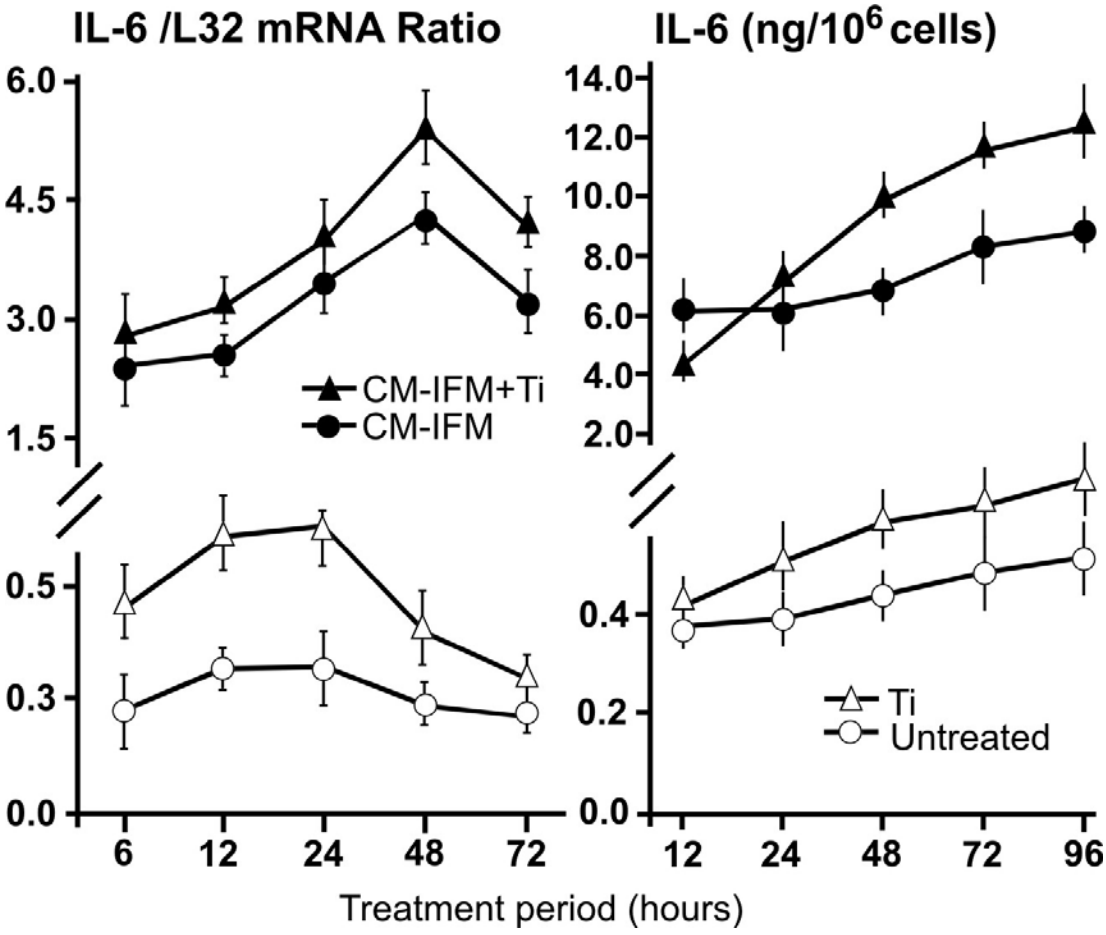

Figure 9C. Time-dependent responses of interface membrane fibroblasts to stimulation with conditioned media from interface membranes (CM-IFM) in the presence or absence of titanium (Ti) particles (0.075% [v/v]). Left panel summarizes the expression of mRNA for IL-6, and right panel shows levels of the corresponding proteins secreted into the medium. IL-6 levels represent the amount of proteins actually secreted (their "original" concentrations in the CM-IFM [incubated without fibroblasts] have been subtracted). Note the nanogram levels of IL-6. Values are the mean ± SEM of triplicate wells from 3–5 independent experiments. See Figure 6 for other definitions.

M-CSF, OPG and RANKL expression by interface membrane fibroblasts in response to stimulation

Fibroblasts of interface membranes expressed mRNA for VEGF, M-CSF (**Figure 6**), OPG and RANKL (**Figure 10**) in response to treatment with conditioned media from interface membranes or treatment with conditioned media from interface membranes plus titanium.

These cells also spontaneously secreted/shed the 24 kDa soluble form of RANKL, and expressed the 48 kDa membrane-bound form of RANKL (32), especially after stimulation (**Figure 10**).

Since both OPG (a decoy receptor of RANKL) and RANKL were detectable in conditioned media from interface membranes (results not shown), and immunolocalized in different cell types of the interface membrane (21,33), we were interested in the capacity of interface membrane fibroblasts to express RANKL. Interface membrane fibroblasts were stimulated with conditioned media, IL-1β or TNF-α, and RANKL expression was detected by flow cytometry (**Figure 11**).

Of note, while the expression of OPG peaked after 12 hours of stimulation and then significantly declined, the expression of RANKL continuously increased in a time-dependent manner (**Figure 10**). Thus, these osteoclastogenic factors (M-CSF and RANKL) were secreted by fibroblasts in response to activation by proinflammatory cytokines, and their levels were further increased in the presence of titanium particles. The combination of conditioned media from interface membranes and titanium particles had a synergistic effect on the expression of RANKL (**Figure 10**).

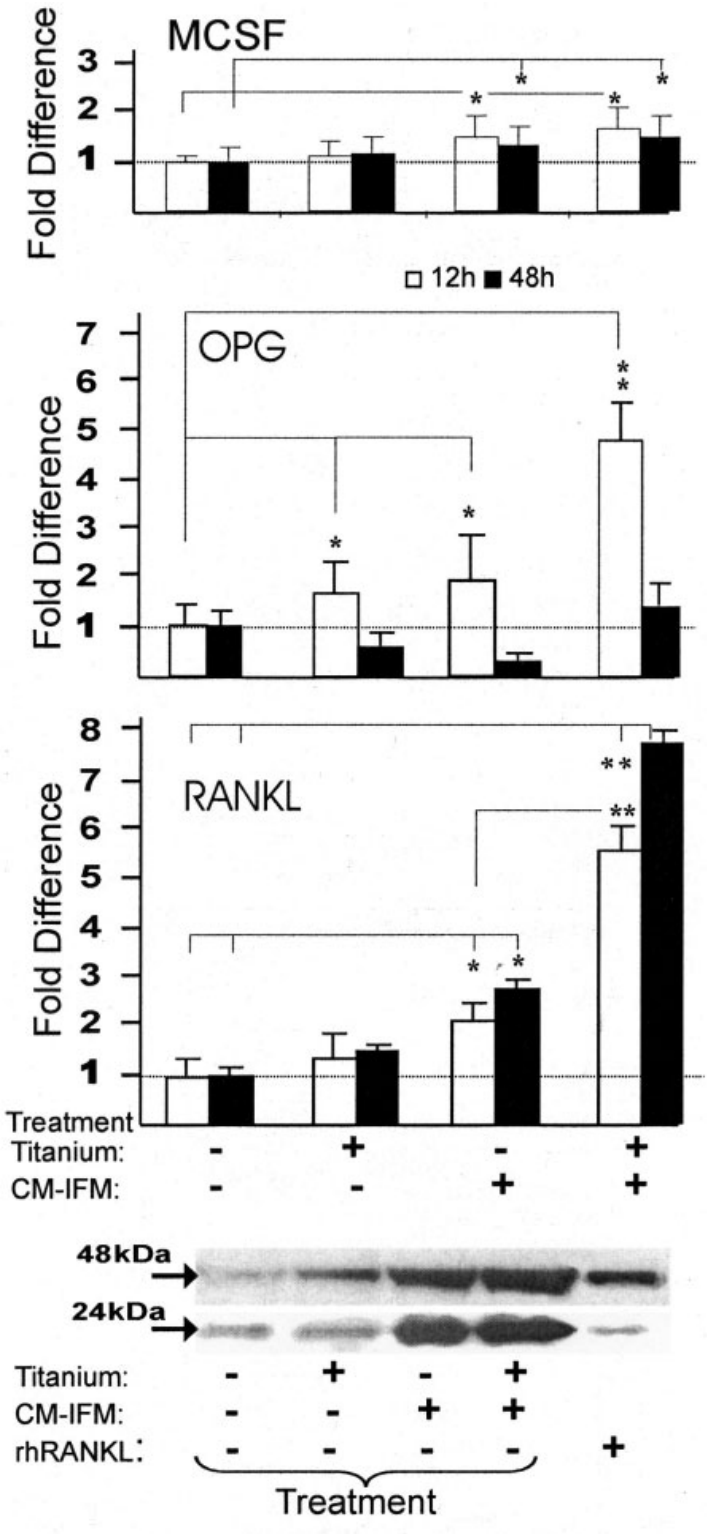

Figure 10. Expression of M-CSF, osteoprotegerin (OPG), and RANKL by interface membrane fibroblasts after 12- or 48-hour treatment with titanium particles and/or conditioned media from interface membranes (CM-IFM). Expression of mRNA for M-CSF was measured by RNase protection assay, and expression of mRNA for OPG and RANKL was measured by real-time polymerase chain reaction. Values are the mean and SEM of 6–12 measurements. ∗ = $P < 0.05$; ∗∗ = $P < 0.01$. Representative Western blot panels show secreted/shed RANKL in medium harvested from fibroblast cultures (24-kDa band), and membrane-bound RANKL from fibroblast cell lysates (48-kDa band). Recombinant human RANKL (rhRANKL) was used as a positive control.

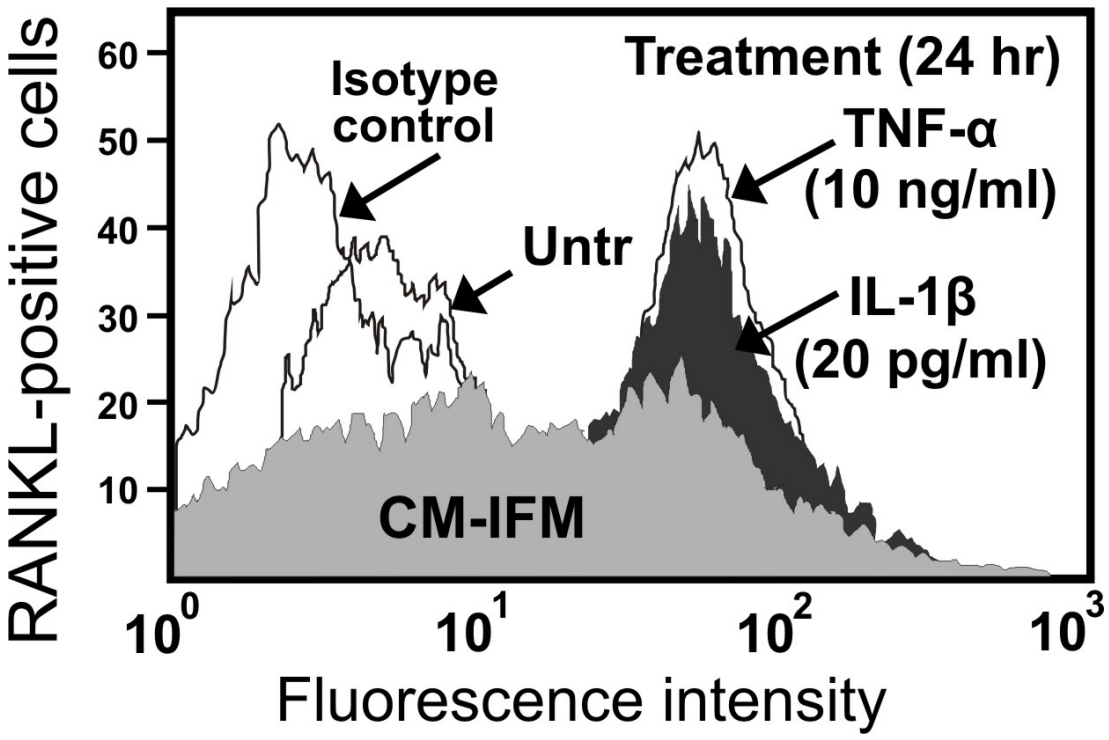

Figure 11. Expression of RANKL by interface membrane fibroblasts after treatment with TNF-α, IL-1β, or conditioned media from interface membranes (CM-IFM).
The panel summarizes flow cytometry results, when interface membrane fibroblasts were stimulated for 24 hours (as indicated) or left untreated (Untr), and then stained with mouse monoclonal antibody (clone 70513; R&D Systems) for RANKL expression. See Figure 6 for other definitions.

Induction of osteoclastogenesis by fibroblast-derived factors in the presence of M-CSF

Since conditioned media from interface membranes, especially in the presence of titanium wear debris, induced significant expression of RANKL by fibroblasts, we established a coculture of human interface membrane fibroblasts and bone marrow-derived stromal cells. These bone marrow-derived stromal cells differentiated to multinucleated TRAP+ cells in the presence of M-CSF and RANKL (**Figure 12A**), but not in the absence of either of these compounds.

Similarly, bone marrow-derived stromal cells that had been cocultured with titanium-stimulated interface membrane fibroblasts (in the presence of M-CSF) differentiated into TRAP+ multinucleated cells (**Figure 12B**), but bone marrow–derived stromal cells never differentiated into TRAP+ multinucleated cells in unstimulated fibroblast cultures or in the absence of M-CSF (results not shown).

Interface membrane fibroblasts activated with either conditioned media from interface membranes (cytokines) or titanium wear debris continuously expressed RANKL, which could then induce osteoclastogenesis in the presence of M-CSF.

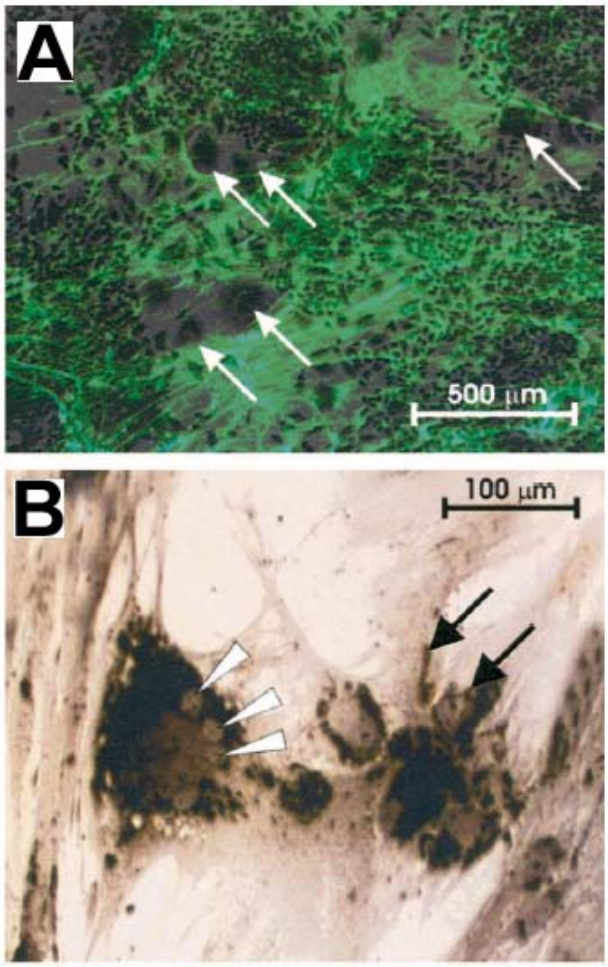

Figure 12. Interface membrane fibroblasts cocultured with human bone marrow cells.
A, Interface membrane conditioned media–stimulated interface membrane fibroblasts and bone marrow–derived stromal cells cocultured for 12 days in the presence of 50 ng/ml macrophage colony-stimulating factor (M-CSF). Fibroblasts were stained first for RANKL with FITC-labeled antibody. **Arrows** show multinucleated cells positive for tartrate-resistant acid phosphatase (TRAP), identified on a hematoxylin-counterstained slide. The 2 corresponding images were overlaid to reconstitute the original coculture condition.
B, Fourteen-day coculture of interface membrane fibroblasts and human bone marrow cells. After 10 passages, fibroblasts ($5X10^4$ cells/well in a 24-well plate) were treated with titanium particles (0.075% [v/v]) as described in Materials and Methods. Other culture wells contained either fibroblasts or bone marrow cells, or both, with or without titanium pretreatment or M-CSF. Multinucleated TRAP+ cells were identified only in wells that contained titaniumpretreated fibroblasts, bone marrow cells, and M-CSF. **Arrowheads** show 3 nuclei of a multinucleated TRAP-stained cell; **arrows** show phagocytosed titanium particles, which are present in both fibroblasts and multinucleated cells.

DISCUSSION

Approximately 30% of periprosthetic soft tissue (interface membrane) is composed of fibroblasts (3,34,35), and these cells have the highest proliferation rate in the interface membrane (16,36), indicating their activated state (37). Proliferation of fibroblasts and vascular endothelial cells in the periprosthetic tissue reflects active tissue remodeling, wherein interface membrane soft tissue replaces the resorbed bone around the prosthetic device. In this particular milieu, different cells and particulate wear debris maintain an endless activation stage, leading to the loosening and failure of joint arthroplasties, which is frequently accompanied by clinically evident periprosthetic osteolysis.

In previous studies we and others showed that IFM fibroblasts were highly activated *in situ* (especially in the bone resorbing areas) (4,36,37), and produced factors which suppressed major osteoblast functions (11). We have chosen human fibroblasts of the IFM for study since, other than tissue macrophages, this is the predominant cell type in the periprosthetic soft tissue (1-4). In order to reproduce the *in vivo* conditions as closely as possible, particles of approximately the same size distribution as the wear debris present in periprosthetic tissues were used to simulate fibroblasts (6,43,45). In this study we focused upon fibroblast activation *in vitro* to monitor how these cells might be involved in pathological bone resorption.

We have shown, by RPA, the overexpression of several "osteoclastogenic" factors (VEGF, MCP-1, M-CSF, COX-1, COX-2, and LIF) by human interface membrane fibroblasts. In addition, real-time quantitative PCR demonstrated overexpression of RANKL and OPG. Cells of this periprosthetic soft tissue, including fibroblasts, are under strong activation pressure due to continuously generated particulate wear debris, which maintains a chronic state of inflammation (26,36,38,39).

Fibroblasts are actively involved in this detrimental process in that 1) they are continuously stimulated by both prosthetic wear debris and cytokines/growth factors produced by activated macrophages, osteoblasts, and fibroblast-secreted products, 2) they suppress osteoblast functions, and 3) they directly or indirectly contribute to osteoclast activation (19,21,22,33,40) (**Figures 12A and B**).

To gain insight into the complex mechanisms taking place in the periprosthetic space and to understand how these fibroblasts are involved in osteolysis, we (in the present study) and others (21,22,34,39,41,42) have used fibroblasts from different sources (synovial tissue of normal and pathologic joints, and interface membranes) and tested their responsiveness under different conditions. In this study, we examined the responsiveness of human fibroblasts of different origins to titanium particles and conditioned media harvested from synovial tissue from normal and rheumatoid joints or from interface membranes. Using the same conditioned media from interface membranes, stimulation of fibroblasts from normal synovial tissue (up to 3–5 passages) was less effective than stimulation of fibroblasts isolated from rheumatoid synovium or interface membranes, although the difference was not significant. In contrast, stimulation with conditioned media from normal synovial tissue had minimal or no effect on fibroblasts isolated from either normal or pathologic (e.g., interface membrane) tissue. Thus, while the *in vitro* response levels of fibroblasts derived from either a normal or an inflammatory milieu were highly comparable after a few passages, cells from inflamed tissue (rheumatoid synovium or interface membrane) produced significantly more bioreactive compounds *in vitro* than cells obtained from normal synovial tissue.

To mimic the *in vivo* conditions of periprosthetic pathologic bone resorption as closely as possible, we stimulated interface membrane fibroblasts with titanium particles and/or conditioned media from interface membranes. In order to reproduce *in vivo* conditions, particles of approximately the same size distribution as the wear debris present in periprosthetic tissue (6,43–45) were used to stimulate fibroblasts. In general, fibroblasts from interface membranes with various degrees of osteolysis responded very similarly after 4–6 passages, but the variability of cytokine/chemokine concentrations in collected conditioned media (**Figure 7C**) had a more diverse effect on fibroblast activation. Therefore, we selected a model system in which we could compare activation events in several fibroblast lines, using conditioned media from a number of interface membranes with high levels of TNF-α, IL-1β, IL-6, IL-8, MCP-1, and VEGF, factors that are involved in osteoclastogenesis.

We found that MCP-1 was as good a marker of fibroblast activation as IL-6, and that both of these secreted compounds had an effect on osteoclast activation, although this effect was indirect (46). While fibroblasts express both tumor necrosis factor receptor p55 (TNFR p55) and TNFR p75 (41,46) and respond to TNF-α stimulation (**Figure 8**), neither synovial nor interface membrane fibroblasts produced TNF-α in response to stimulation with titanium particles or conditioned media from interface membranes (**Figure 6**). In contrast, exogenous TNF-α significantly up-regulated IL-1β, IL-6, and M-CSF expression, and even more dramatically up-regulated MCP-1 and RANKL expression, in both interface membrane fibroblasts (**Figures 8** and **11**) and synovial fibroblasts (data not shown). Thus, fibroblasts, via TNFR p55, might be involved in both RANKL-dependent and RANKL-independent osteoclastogenesis (47), and the therapeutic effects of various anti-TNF therapies (48,49) may, at least in part, be associated with these mechanisms. This observation suggests that activated fibroblasts, upon exposure to particles and/or proinflammatory cytokines (TNF-α and IL-1β) produced by adjacent cells in the interface membrane, secrete large amounts of chemokines (such as MCP-1 and IL-8), thereby recruiting cells of the myeloid lineage.

Among the fibroblast activation markers induced by either TNF-α, IL-1β, particulate titanium, or conditioned media from interface membranes, the expression of membrane-bound RANKL, a potent osteoclastogenic mediator, together with soluble forms of RANKL (24 kDa and lesser amounts of 27.6 kDa) (**Figure 10-11**) was detected. In addition, activated fibroblasts secreted VEGF (**Figure 6** and **7C**) and M-CSF (**Figures 6** and **10**), which are also needed for osteoclastogenesis (46,50–52). Therefore, many of the osteoclastogenic factors detected in the interface membrane (21,53,54) might derive from activated fibroblasts (**Figure 7**), but they are less likely derived from RANKL+ activated T or B cells (46), which are rare in the interface membrane.

RANKL-expressing osteoblasts, and especially fibroblasts that express RANKL, are purportedly primarily responsible for osteoclast differentiation and activation (21,22,33,46,54) and subsequent periprosthetic osteolysis (21,53,54). Osteoblasts, however, are rarely seen in the osteolytic areas (4). In contrast, fibroblasts and macrophages are present adjacent to osteoclasts (4), and because activated fibroblasts secrete RANKL, VEGF, and M-CSF, it may well be that the fibroblast is the key cell type moderating osteoclastogenesis in periprosthetic osteolysis.

To mimic this *in vivo* condition *in vitro*, we used interface membrane conditioned media–stimulated or particulate titanium–stimulated interface membrane fibroblasts, cells that express RANKL, to induce the formation of multinucleated TRAP+ osteoclast-like cells from human bone marrow stromal cells in the presence of M-CSF (**Figure 12**). Similar mechanisms have been hypothesized to take place in bone-resorbing rheumatoid synovium (41,46).

Taken together, the present results indicate that macrophage activation and fibroblast activation are "natural" processes in the interface membrane and rheumatoid synovium, and the effect of fibroblast activation on osteoclastogenesis and subsequent bone resorption may be as potent and critical as that of macrophage activation. In addition, activated fibroblasts produce large amounts of bone-resorbing metalloproteinases accompanied by reduced secretion of tissue-specific metalloproteinase inhibitors (19), which, together with fibroblast-induced suppression of osteoblast function (11), suggests that fibroblasts have a significant role in the pathogenesis of periprosthetic osteolysis.

Of marked importance for future investigation will be the careful analysis of retrieved clinical specimens to better characterize the nature of the particulate wear debris. The next challenge will be for the biomaterials engineers to produce comparable materials for testing in *in vitro* and *in vivo* models so that the biological properties of these materials can be defined.

The importance of mechanical factors and material properties in the pathogenesis of aseptic loosening of implants has been well established, and these processes clearly are essential elements in the complex sequence of events that accompany the failure of an implant. Studies provide important insights into how biological processes contribute to these events. Although major efforts need to be continued to reduce the adverse effects of mechanical factors and to decrease the potential for materials failure, an awareness of these biological processes could lead to the development of additional therapeutic approaches that will prolong the survival of implants. The application of experimental models may allow investigators to evaluate the potential efficacy of pharmacological agents in terms of their ability to slow the loosening process by selectively inhibiting the mediators of bone resorption that is induced in response to particulate debris. Furthermore, this information could contribute to the development of alternative methods of fixation of implants that reduce the tendency of the implant to generate particulate debris.

Alternatives to cement as well as the introduction of different materials and designs for the fabrication of implants that have less biological activity, even when particles of the prosthesis are released, are potential outcomes of these investigations. It is anticipated that an increasing awareness of these biological factors will be used in conjunction with the knowledge of mechanical and materials factors, with the ultimate goal of delaying or preventing aseptic loosening.

THESIS

RESULTS

Fibroblasts phagocytosed particulate wear debris, and responded to cytokine/chemokine stimulation. The most dominant compounds measured in CM of IFM were tumor necrosis factor-α (TNF-α), monocyte/macrophage chemoattractant protein-1 (MCP-1), interleukin-1β (IL-1β), IL-6, IL-8 and vascular endothelial growth factor (VEGF). The most prominent upregulated genes, and secreted proteins by fibroblasts in response to stimulation were: matrix metalloproteinase-1, MCP-1, IL-1β, IL-6, IL-8, cycloxygenases (Cox-1 and Cox-2), leukemia inhibitory factor-1 (LIF-1), transforming growth factor β-1 (TGF-β1), TGFβ receptor-I and VEGF.
In addition, IFM fibroblasts expressed RANKL (receptor of activated nuclear factor-kappa B ligand) and OPG (osteoprotegrin) in response to CM-, TNF-α-, or IL-1β-stimulation. Particulate titanium stimulated RANKL+ fibroblasts co-cultured with bone marrow cells induced osteoclastogenesis.

CONCLUSION

IFM fibroblasts respond directly to particulate wear debris, possibly via phagocytosis, expressing proinflammatory cytokines and RANKL.
Fibroblasts activated by either particulate wear debris or proinflammatory cytokines, are capable of expressing a wide array of osteoclastogenic factors which are involved in the detrimental processes of the periprosthetic tissue remodeling and osteolysis.
These cells are actively involved in diminished bone remodeling and pathological (e.g. periprosthetic) bone resorption.

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ACKNOWLEDGEMENTS

Herein, I wish to thank everyone providing me encourage, help and support: members and orthopedic surgeons, especially Mitchell Sheinkop M.D. of Rush University Medical Center for collecting tissue samples; Alison Finnegan, Katalin Mikecz, Tibor T. Glant, Nadim J. Hallab, István Gál, Jian Zhang, Yanal Murad, Miklós Tunyogi-Csapó and Csaba Vermes for their valuable discussions; and Sonja Velins for her technical help during the performance of this study.

I am grateful for the instruction and scientific support of my teacher László Hangody, Head of Department of Orthopaedics, Uzsoki Hospital, Budapest.

I would also like to acknowledge the help and assistance of all members of the Institute of Musculoskeletal Surgery, University of Pécs, with special regards to my chief László Vámhidy and my leader Árpád Bellyei for his great support and for inspiring my studies.

Thanks to my Melinda, my family and my friends for the love and support during all my studies and research work.

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