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

Ph.D. thesis

Tamás Koreny M.D.

Head of doctoral school:	Prof. Sámuel Komoly M.D., DSc.
Leader of program:	Prof. Árpád Bellyei M.D., DSc.
Tutor:	Csaba Vermes M.D., PhD.

UNIVERSITY OF PÉCS
CLINICAL CENTER
INSTITUTE OF MUSCULOSKELETAL SURGERY
DEPARTMENT OF TRAUMATOLOGY AND HAND SURGERY

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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)

1. INTRODUCTION

Periprosthetic osteolysis following total joint arthroplasty (TJA) is a major clinical problem in both cemented and cementless reconstructions. 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 or bone/prostheses interface. 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 pen-implant osteolysis has been recognized as a major problem contributing to aseptic loosening of total hip prostheses that have been inserted without 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).

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

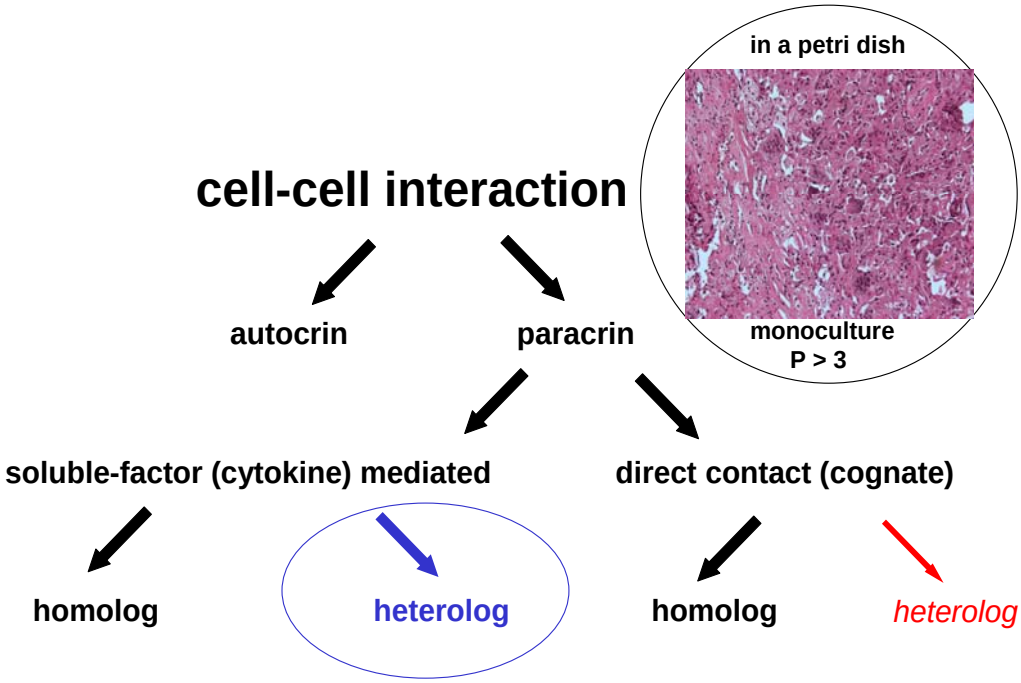


Figure 1. Cell-cell interactions *in vivo*. Black scripts represent previous *in vitro* models. Black+circulated scripts represent our improved *in vitro* model. Black+circulated+*italic* scripts would represent a perfect model.

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.

Osteoblasts also phagocytose particles, and particulate phagocytosis significantly increases the secretion of TNF-α, IL-6, and the cell surface expression of RANKL, simultaneously suppressing procollagen E1[I] transcription.

Information on the fibroblast (dominant cell type in the IFM) response to particle debris is less extensive. 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. 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.

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 were used to stimulate fibroblasts.

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.

2. AIMS

The aim of this study was to determine what role fibroblasts – most dominant cell type in the IFM - play in the pathogenesis of periprosthetic osteolysis associated with particle wear debris.

We wanted to establish an improved *in vitro* model to mimic the *in vivo* conditions of periprosthetic pathologic bone resorption as closely as possible (**Figure 1**).

We wanted to proove that fibroblasts respond to stimulation with particulate wear debris and/or else environmental factors obtained from pathologic tissues, and whether these activation is stronger, moreover activated fibroblasts express more effective compounds, than in previous *in vitro* models.

We wanted to verify that fibroblasts produce the highest amounts of cell mediators which are involved in bone resorption.

3. MATERIALS AND METHODS

3.1 Particles and chemicals

Commercially pure small-sized titanium particles (<3 μm) were sequentially treated and then washed with sterile, endotoxin-free phosphate buffered salt solution (PBS; pH 7.2). Endotoxin/lipopolysaccharide contamination of particles was excluded by *Limulus* amoebocyte cell lysate assay. Particles were autoclaved, sonicated and then sedimented 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, a 0.075% (volume/volume: v/v) Ti suspension contained approximately 6 x 10^7 particles/ml.

3.2 Patients and tissue samples

The collection of human samples from joint replacement and revision surgeries, and the use of bone marrow aspirates, were collected in consent with the patient.

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]).

3.3 Explant cultures and conditioned media (CM)

Tissue samples in sterile containers of DMEM and 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. Approximately 0.5g wet synovial or interface membrane tissue was cultured in 2.5 ml DMEM containing 5% fetal bovine serum (FBS), antibiotic/antimycotic solution, which was supplemented with 50 µg/ml gentamicin. Tissue samples were distributed, and 90% of the medium was replaced daily for a total of seven days. 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 (medium control) was also incubated, harvested, centrifuged, and stored in the same manner as all other conditioned media.

3.4 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. Dissociated cells were washed with PBS and plated in Ø10cm petri dishes 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.

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 using anti-CD90 (Thy-1) monoclonal antibody (mAb) and by immunohistochemistry using fluorochrome-labeled mAb 5B5 to F-subunit of propyl-4-hydroxylase. Freshly isolated fibroblasts from IFM contained particles, 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, 99-100% of the cells were CD90+ fibroblasts.

3.5 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 (medium control) or conditioned media with or without titanium particles for different time periods. Data in this report summarize the responses of 2–4 independent fibroblast lines isolated from interface membranes.

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. 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). Prior to the experiments on fibroblast stimulation, conditioned media from interface membranes were individually pretested for 48 hours for cytokine production. These conditioned media from interface membranes were pooled and filtered. Each of these pools were then tested 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.

3.6 *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, except that these bone marrow-derived progenitor cells were used for osteoclast formation. Bone marrow cells were transferred to slides 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 recombinant macrophage colony stimulating factor (M-CSF) for 8-14 days. Fibroblasts were stained for CD90 and RANKL, fluorescent images were stored, and then restained for tartarate-resistant acid phosphatase (TRAP). Multinucleated (> 4 nuclei per cell) TRAP+ cells were counted. Unstimulated interface membrane fibroblasts, interface membrane fibroblasts stimulated with M-CSF alone, or 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) were used as positive controls. Additionally, fibroblasts were plated 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 (0.8×10^6/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.

3.7 RNA isolation and RNase protection assay (RPA)

Fresh tissue samples (~0.2-0.4g), and tissue samples cultured for 7 days, were homogenized, homogenate was centrifuged, and RNA was extracted by TRIzol reagent. TRIzol was also used to isolate total RNA from cultured fibroblasts before and after treatments. The amount of RNA was determined using a RiboGreen RNA quantification kit.

RPA was performed on 8 μg of RNA using the Riboquant Multiprobe RNase Protection Assay System. In addition to commercially available templates, 2 custom-made RPA templates were purchased. 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).

The 32P-labeled samples of different lengths were separated on 5% denaturing polyacrylamide/8M urea gel. Radioactivity of the samples was measured and analyzed by scanning densitometry on a Storm PhosphorImager. We found a high correlation (> 95%) between the amount of ribosomal RNA and the housekeeping genes L32 and GAPDH. However, L32 expression was more consistent in titanium-stimulated fibroblast cultures, and all samples were normalized to L32.

3.8 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. The detection was carried out by measuring the binding of fluorescent SYBR Green-I to double stranded DNA. The PCR reactions were carried out by 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). 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. The RANKL and OPG signals were normalized against the quantity of L32 and 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.

3.9 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.

3.10 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 containing protease inhibitors, phosphatase inhibitors for 1 hour at 4^{O}C.

Cell lysates were cleared by centrifugation, and protein was separated by gel electrophoresis. Proteins were transferred onto membranes, and membranes were blocked with milk, and stained with anti-RANKL mAb or rabbit polyclonal antibody. The 24 and 48 kDa bands were identified with recombinant human RANKL.

For flow cytometry, fibroblast cultures were harvested, and then washed. Cells were incubated with 10 ng/μl of mouse anti-human-RANKL mAb for 1 hour at 4^{O}C, followed by biotin-labeled goat anti-mouse Ig antibody (10 ng/μl). The reaction was developed with streptavidin-phicoerythrin. Samples were fixed in 2% formalin and then analyzed by FACSCalibur using CellQuest software. An IgG1 isotype control mAb was used to determine nonspecific background levels in all experiments.

3.11 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.

4. RESULTS

4.1 Selection of ”bone-resorbing” factors

Synovial tissue samples from normal and rheumatoid joints were compared to those measured in periprosthetic (interface membrane) soft tissues. Although a number of factors were measured, only the results of TNF-α, MCP-1, IL-1β, IL-6, IL-8 and VEGF are shown (**Figure 2**).

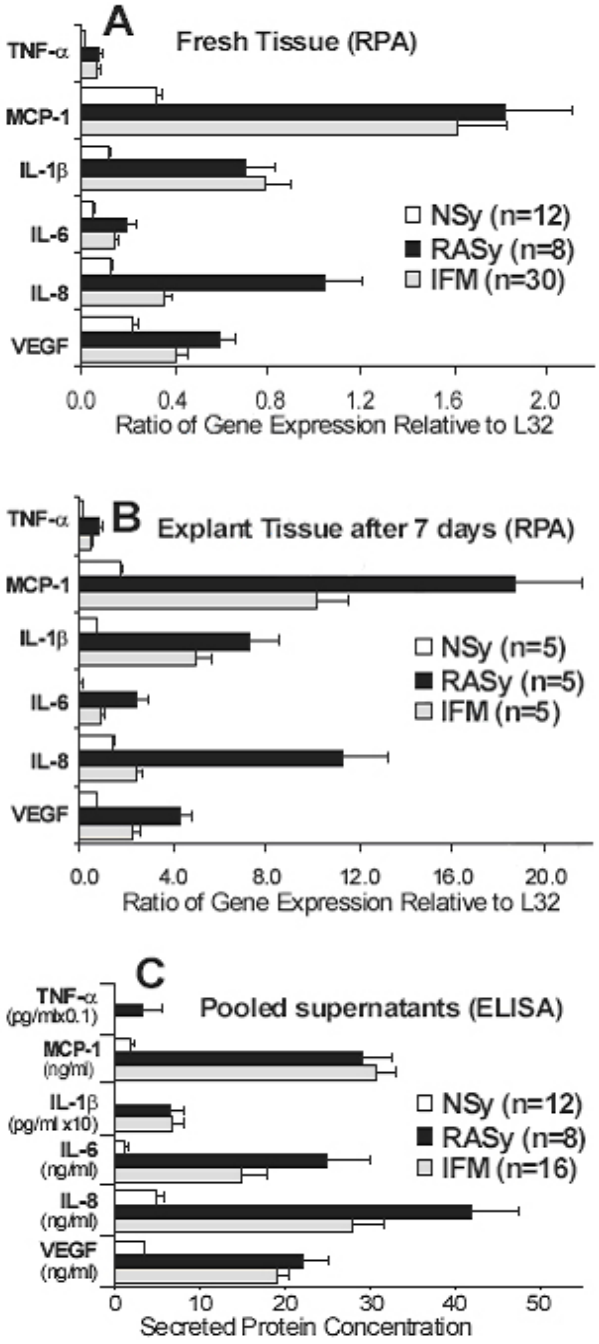


Figure 2. A and **B**, Steady-state mRNA levels measured in normal synovial tissue (NSy), rheumatoid synovial tissue (RASy), and interface membranes (IFM), either freshly isolated **(A)** or after a 7-day culture period **(B)**. **C**, Secreted cytokine, chemokine, and VEGF levels measured in pooled conditioned media of explant cultures.

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

Normal synovial tissue expressed significantly lower message levels for all cytokines, chemokines and growth factors than did either synovial samples from rheumatoid joints or interface membrane tissue samples (**Figure 2A**).

The gene-specific mRNA expression continuously increased until day 7, the final day of explant cultures (**Figure 2B**), when the message levels for all measured genes were approximately 4-10-fold higher than in fresh samples (**Figure 2A**). These findings corresponded to the results of measurement of secreted cytokines and growth factors (**Figure 2C**).

4.3 Selection of fibroblasts and fibroblast activation markers

Originally, we tested fibroblasts of different origins, and IL-1β, MCP-1 and IL-6 seemed to be the most sensitive and consistent markers of fibroblast activation. 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. With this cocktail, we observed similar levels of IL-6, IL-1β, and MCP-1 secretion by fibroblasts as were found when conditioned media from pathologic samples were used (**Figure 3**).

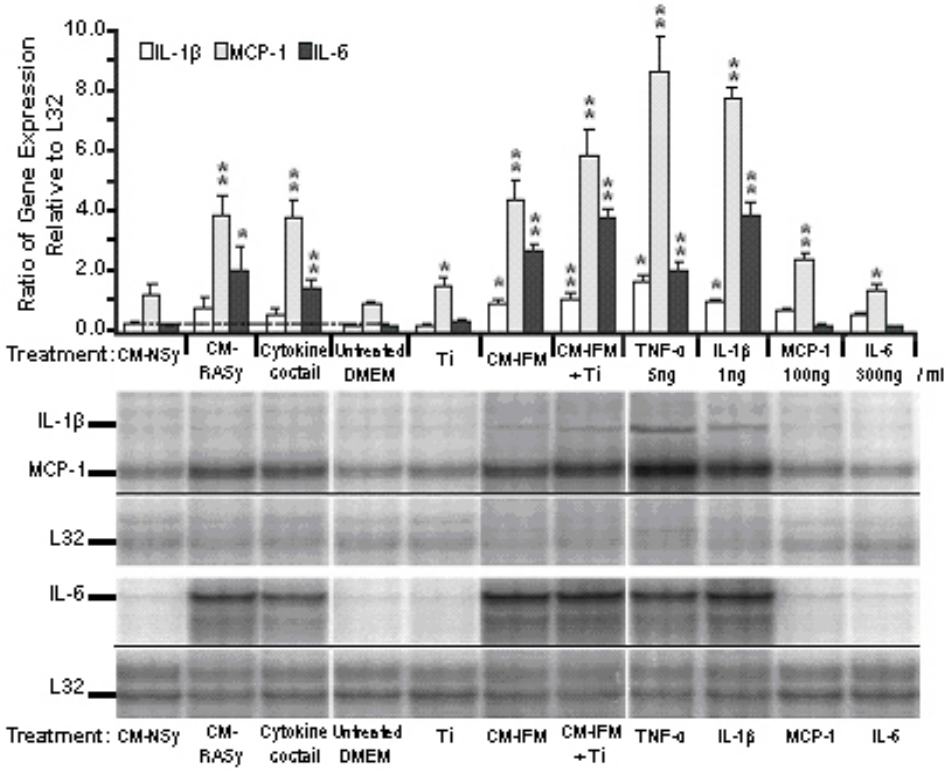
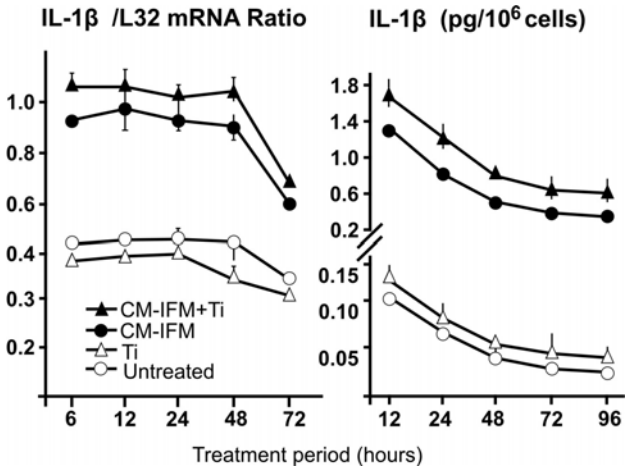


Figure 3. Expression of mRNA for IL-1β, MCP-1, and IL-6 in interface membrane fibroblasts cultured in conditioned media harvested from explant cultures of normal synovial tissue (CM-NSy) and rheumatoid synovial tissue (CM-RASy), interface membranes from loosened implants (CM-IFM), and a "cytokine cocktail" containing TNF-α, IL-1β, IL-6, IL-8, VEGF, and MCP-1.

4.4 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* or *in vitro*, and that fibroblasts responded to stimulation with titanium particles, inflammatory cytokines, and conditioned media (**Figure 3**). 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. 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. 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.

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 4**).



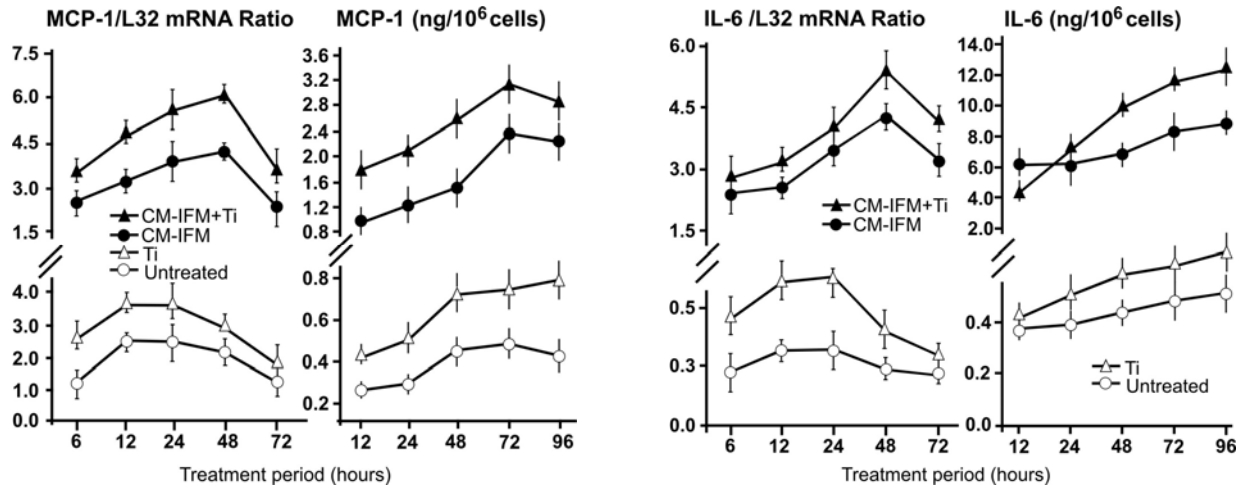


Figure 4. Time-dependent responses of IFM fibroblasts to stimulation with conditioned media from interface membranes (CM-IFM) in the presence or absence of titanium particles (0.075% [v/v]). Left panel summarizes the expressions of mRNA for IL-1β, MCP-1, and IL-6 and right panel shows levels of the corresponding proteins secreted into the medium. Note the nanogram levels of MCP-1 and IL-6.

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

Fibroblasts of interface membranes expressed mRNA for VEGF, M-CSF, OPG and RANKL 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, especially after stimulation. 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. 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 IFM and titanium particles had a synergistic effect on the expression of RANKL (**Figure 5**).

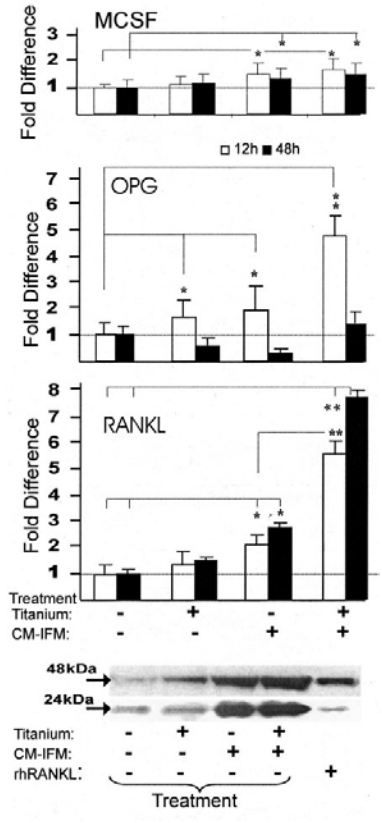


Figure 5. Expression of M-CSF, osteoprotegerin (OPG), and RANKL by interface membrane fibroblasts after 12- or 48-hour treatment with titanium particles and/or conditioned media from interface membranes (CM-IFM). Representative Western blot panels show secreted/shed RANKL in medium harvested from fibroblast cultures (24-kDa band), and membrane-bound RANKL from fibroblast cell lysates (48-kDa band). Recombinant human RANKL was used as a positive control.

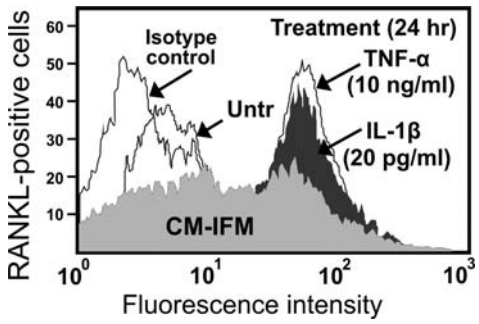


Figure 6. Expression of RANKL by interface membrane fibroblasts after treatment with TNF-α, IL-1β, or conditioned media from interface membranes (CM-IFM). The panel summarizes flow cytometry results, when interface membrane fibroblasts were stimulated for 24 hours (as indicated) or left untreated (Untr),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

Since both OPG and RANKL were detectable in conditioned media from interface membranes, and immunolocalized in different cell types of the interface membrane, we were interested in the capacity of interface membrane fibroblasts to express RANKL. Interface membrane fibroblasts were stimulated with conditioned media, titanium wear debris, IL-1β or TNF-α, and RANKL expression was detected by flow cytometry (**Figure 6**).

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

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, 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, but bone marrow–derived stromal cells never differentiated into TRAP+ multinucleated cells in unstimulated fibroblast cultures or in the absence of M-CSF. 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.

5. DISCUSSION

Approximately 30% of periprosthetic soft tissue (interface membrane) is composed of fibroblasts, and these cells have the highest proliferation rate in the interface membrane, indicating their activated state. Proliferation of fibroblasts 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.

The present results indicate that macrophage activation and fibroblast activation are “natural” processes in the interface membrane, 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, many of the osteoclastogenic factors detected in the interface membrane might derive from activated fibroblasts producing large amounts of bone-resorbing metalloproteinases accompanied by reduced secretion of tissue-specific metalloproteinase inhibitors which, together with fibroblast-induced suppression of osteoblast function, suggests that fibroblast is the key cell type moderating osteoclastogenesis in periprosthetic osteolysis.

2. NOVEL FINDINGS

We determined that activated fibroblasts, upon exposure to particles and/or proinflammatory cytokines produced by adjacent cells in the interface membrane, secrete large amounts of chemokines, thereby recruiting cells of the myeloid lineage. We proved that human fibroblasts are actively involved in periprosthetic osteolysis, as they suppress osteoblast functions, and directly or indirectly contribute to osteoclast activation. We showed that stimulated RANKL+ fibroblasts co-cultured with bone marrow cells induced osteoclastogenesis.

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 and conditioned media harvested from synovial tissue from normal joints or from interface membranes were used to simulate human fibroblasts.

We showed that fibroblasts could phagocytose particles either *in vivo* or *in vitro*, and that fibroblasts responded to stimulation with titanium particles, inflammatory cytokines, and conditioned media. The response was observed at both the transcriptional and the translational levels. We found that MCP-1 was as good a marker of fibroblast activation as IL-6, and fibroblasts secreted nanogram levels of MCP-1 and IL-6 in our improved *in vitro* model.
In contrast to previous *in vitro* models, fibroblasts with combination treatment were able to secrete IL-1β, TGF-β1 and more.

We have shown the overexpression of several osteoclastogenic factors. 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, Cox-1, Cox-2, LIF, TGF-β1, TGFβ receptor-I, RANKL, OPG and VEGF.
Titanium particles and conditioned media from interface membranes exhibited either additive or synergistic effects on secreting nanogram levels of mediators, which are known to be involved in osteoclast maturation and activation.

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