Fibroblast-mediated pathologic bone resorption in ex vivo and in vivo models

Summary of Ph.D. thesis

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Department of Orthopedic Surgery
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ABBREVIATIONS

Ang-1	Angiopoietin 1
APC	Antigen presenting cell
cDNA	Complementary DNA
CD90	cluster of differentiation 90 (fibroblast marker)
CM	Conditioned media
Cox	Cycloxygenase
Ct	Treshold cycle
DMEM	Dulbecco's modified Eagle's medium
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FGF	Fibroblast growth factor
FLS	Fibroblast like synoviocyte
GAPDH	Glyceraldehyde-6-phosphate-dehydrogenase
IFFb	Interfacial membrane fibroblast
IFM	Periprosthetic interfacial membrane
IFN	Interferon
IL	Interleukin
LIF	Leukemia inhibitory factor
MCP	Monocyte/macrophage chemoattractant protein
M-CSF	Macrophage colony-stimulating factor
MMP	Matrix metalloproteinase
NSy	Normal synovium
OA	Osteoarthritis
OPG	Osteoprotegerin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polimerase chain reaction
PG	Proteoglycan
PGIA	Proteoglycan-induced arthritis
qRT-PCR	Quantitative reverse transcriptse polymerase chain reaction
RA	Rheumatoid arthritis
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa B ligand
RANTES	Regulated upon activation normally T-cell expressed and secreted
RASy	Rheumatoid synovial tissue
RASFb	Rheumatoid synovial fibroblast
RPA	Rnase protection assay
SDS	Sodium duodecyl sulfate
SEM	Standard error of mean
SFs	Synovial fibroblasts
TGF	Transforming growth factor
Ti	Titanium
TIMP	Tissue inhibitor of metalloproteinase
Th-cell	T-helper cell
TJA	Total joint arthroplasty
THA	Total hip arthroplasty
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor

1. INTRODUCTION

Osteoclasts and osteoblasts dictate skeletal mass, structure, and strength via their respective roles in resorbing and forming bone. Bone remodeling is a spatially coordinated lifelong process whereby old bone is removed by osteoclasts and replaced by bone-forming osteoblasts. The refilling of resorption cavities is incomplete in many pathologic states, which leads to a loss of bone mass with each remodeling cycle. This resorption process could be generalized net loss (e.g: osteoporosis) or focal destruction (e.g.: RA joint destruction or periprosthetic osteolysis). Bone resorption is dependent on a cytokine known as RANKL (receptor activator of nuclear factor kappa B ligand), a TNF (tumor necrosis factor) family member that is essential for osteoclast formation, activity and survival in normal and pathologic states of bone remodeling. The catabolic effects of RANKL are prevented by OPG (osteoprotegerin), a TNF receptor family member that binds RANKL and thereby prevents activation of its single cognate receptor called RANK. Osteoclast activity is likely to depend, at least in part, on the relative balance of RANKL and OPG. Studies in numerous animal models of bone disease show that RANKL inhibition leads to marked suppression of bone resorption and increases in cortical and cancellous bone volume, density and strength. RANKL inhibitors also prevent focal bone loss that occurs in animal models of rheumatoid arthritis.

Pathologic bone resorption around endoprosthesises is a major issue in orthopedic surgery due to the formation of an agressive inflammatory granulomatous tissue, caused by particulate wear debris, that leads to the loosening of total joint arthroplasties. Rheumatoid arthritis also results focal bone erosions where the inflammatory process targets the articular cartilage, the bone at the joint margins, as well as periarticular and subchondral bone, and originated from the inflamed synovium. The characteristics of the inflamed tissue around the destructive bone resorbing zone shows several similarities between the two different pathologies. The dominant cell type at the sites of invasion into the adjacent bone is synovial fibroblast and in both tissues similar pro- and anti-infalmmatory cytokines can be detected. During the infalmmatory process the thickness of the synovial like tissue is increasing according to the influx and proliferation of inflammatory cells as well as the increased proliferation and survival of resident cells, although the terminal layer of the tissue matrix predominantly contains 10-15 cell layers of fibroblast like cells. The inflamed tissue also shows an increased neoangiogenesis, facilitating the influx of inflammatory cells. Overall the pathological processes of osteoclastogenesis, dysregulated bone formation, granulomatosus tissue formation and neovascularization are simultaneous and overlapping events that cannot be separeted. However T cells and other inflammatory cells are rarely seen at the site of bone resorption, either in RA, in corresponding animal models, or in periprosthetic osteolysis; rather fibroblast-like and macrophage-like cells with osteoclasts, and less frequently osteoblasts occupy the resorbed areas of bone. In this thesis we were focusing on the role of synovial fibroblasts in this mandatory process of pathologic bone resorption which leads to prosthesis looseneing and rheumatoid joint destruction.

2. AIMS AND HYPOTHESIS

To gain more insight into the mechanisms of pathologic bone resorption and angiogenesis that takes place in arthritic joints and between the prosthesis/bone inetrface, and to understand how synovial fibroblasts in a cytokine-rich environment are involved in these processes, we aimed to prove the following hypotheses.

» *During subsequent joint destruction and prosthesis loosening synovial fibroblasts are actively contribute to bone resorption, and neovascularization by expressing a wide array of osteoclastogenic and angiogenic factors. These compaunds play an important role in the detrimental processes of rheumatoid arthritis and periprosthetic osteolysis.*

To reach our goals, we performed the following experimental studies:

Study I. To determine whether proinflammatory cytokine treatment or the complete absence of select cytokines modulates the expression of major osteoclastogenic factors (RANKL and OPG) in synovial fibroblasts

- We performed in vitro and in vivo experiments using *human* and *mouse* synovial fibroblasts and different cytokine milieus. We compared in vitro expression of RANKL and OPG in normal versus RA and IFM synovial fibroblasts from human origin
- Then the same experiments were repeated using fibroblasts from normal and arthritic mouse knee joints.
- Finally, utilizing our extensive experience with a mouse model of arthritis (proteoglycan [PG]-induced arthritis; PGIA), we applied in vitro conditions in vivo, when the antiinflammatory and antiosteoclastogenic cytokines IFNγ and IL-4 and the osteoclastogenic cytokine IL-17 were absent, in naive and arthritic gene-deficient animals.

Study II. To examine the role of synovial fibroblasts and fibroblast derived-growth factors is periprosthetic angiogenesis

- The purpose of the second study was to determine whether synovial fibroblast plays a key role in angiogenesis within the periprosthetic tissues.
- We evaluated this by measuring major angiogenic factors produced by synovial fibroblasts (IFFb and RASF) in response to particulate wear debris and proinflammatory cytokines.

3. MATERIALS AND METHODS

3.1.1. Chemicals and cytokines

All chemicals, unless otherwise indicated, were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Chicago, IL). Human and mouse recombinant proteins for fibroblast treatments such as TNFa, IL-1b, IFNg, IL-17, and IL-4 were purchased from R&D Systems (Minneapolis, MN) or Sigma.

3.1.2 Fibroblast isolation, and human synovial cultures

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. 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 using anti-CD90 (Thy-1) monoclonal antibody (mAb) and by immunohistochemistry in 8-well chamber slides (Nalgene) using fluorochrome-labeled mAb 5B5 to F-subunit of propyl-4-hydroxylase. Thus, these cells were considered to be synovial (normal, interfacial memrane or rheumatoid) fibroblasts (i.e., FLS).

3.1.3 Statistical analysis

Descriptive statistics were used to determine group means and standard error of the mean. The Pillai's trace criterion was used to detect multivariate significance. Subsequently, Mann Whitney U-test was performed to compare the results of experimental groups. The level of significance was set at $p<0.05$. All statistical analyses were performed using computer-based statistical software (SPSS/PC+ v 15 SPSS Inc, Chicago, IL).

3.2. Methods for human and mouse osteoclastogenesis study (Study I)

3.2.1. Mice, immunization, and mouse synovial fibroblast cultures.

All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Rush University Medical Center. Adult BALB/c mice were purchased from the National Cancer Institute. IL-$4^{-/-}$ and IFN$g^{-/-}$ ice on a BALB/c background were purchased from The Jackson Laboratory (Bar Harbor, ME), and IL-$17^{-/-}$ mice, also on a BALB/c background, were provided by Dr. Y. Iwakura (University of Tokyo). For initiation of PGIA, wild-type and gene-deficient mice were injected 2-3 times intraperitoneally with 100 μg of human cartilage PG (aggrecan) in dimethyldioctadecylammonium bromide adjuvant at 3-week intervals. Severe arthritis developed 7-10 days after the second PG injection in all IL-$4^{-/-}$ mice and in many of the wild-type and IL-$17^{-/-}$ BALB/c

mice. Nonarthritic wild-type and IL-17$^{-/-}$ mice received a third injection, as did the IFNγ$^{-/-}$ mice, in which a relatively mild arthritis developed only after the third PG injection. Arthritis ultimately developed in all wild-type and gene-deficient mice, and the degree of inflammation was assessed visually. The knee joints of age-matched naive and PG-immunized wild-type and gene-deficient mice were used for synovial fibroblast isolation, as previously described for human cultures. Fibroblasts were used for experiments after 4-5 passages, when the cultures showed >98% synovial fibroblast phenotype, as described for human FLS. For histologic assessment, the hind paws were fixed in formalin, decalcified, and embedded in paraffin.

3.2.2. Treatment of synovial fibroblasts with cytokines.

Confluent cultures of fibroblasts were subjected to serum deprivation in Dulbecco's modified Eagle's medium containing 0.5% fetal bovine serum, for 24 hours. This medium was replaced with fresh medium containing the appropriate cytokine concentration (determined in preliminary experiments for both human and mouse naive and arthritic fibroblast cultures). The in vitro responses of human and mouse FLS to cytokines were pretested using TNFα (1.25-10 ng/ml), IL-1β (0.2-5 ng/ml), IL-4 (2-10 ng/ml), IFNγ (1-15 ng/ml), and IL-17 (25-100 ng/ml) in dose-response and time-curve experiments. In the final experiments (which are described in this report), 5 ng/ml of TNFα, 1 ng/ml of IL-1β, and 25 ng/ml IL-17 were used alone or in combination with 5 ng/ml of IL-4 or 5 ng/ml of IFNγ.

3.2.3. RNA extraction, complementary DNA (cDNA) synthesis, and real-time quantitative polymerase chain reaction (qRT-PCR).

Total RNA was extracted from human and mouse synovial fibroblasts with TRIzol reagent, following the manufacturer's protocol. The RNA was quantified with a RiboGreen Quantitation Kit, and the quality of RNA was determined by formamide agarose gel electrophoresis. Real-time quantitative PCR analyses of RANKL, OPG, and GAPDH were performed on fibroblast-derived reverse-transcribed RNA using the TaqMan Gene Expression Assay. Serial dilutions ranging from 1:1 to 1:8 of cDNA were amplified using GeneAmp Fast PCR Master Mix. The housekeeping gene GAPDH was used as a reference in each sample.

3.2.4. RANKL, OPG protein, and cytokine enzyme-linked immunosorbent assays (ELISAs).

Conditioned media of human and mouse fibroblast cultures and of mouse sera and paw extracts were analyzed for soluble RANKL (sRANKL), OPG, TNFa, IL-1b, IL-6, IL-17, IL-4, and IFNg using DuoSet ELISA Development kits (R&D Systems) according to the manufacturer's instructions. After several commercially available ELISA kits were tested for specificity and sensitivity, human sRANKL ELISA kits were purchased from BioVision. The RANKL, OPG, and cytokine concentrations (ng) in

conditioned media were normalized to 1 million fibroblasts, and ng/ml in serum, or ng/mg protein in mouse paw extracts. We also determined the complex form of sRANKL/OPG using cross-capture ELISA systems. For example, if anti-OPG capture antibody was coated, the OPG-RANKL complex, i.e., OPG-bound sRANKL, was detected with anti-RANKL detection antibody and vice versa. Overall, although only 8-10% of OPG was in complex, approximately half the amount of sRANKL was bound to OPG.

3.3. Methods for human angiogenesis study (Study II.)

3.3.1. 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. Approximately 0.5g wet synovial or interface membrane tissue was cultured in 2.5 ml DMEM containing 5% endotoxin-free fetal bovine serum, 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. 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. Eventually the same patient population, tissues, explant and fibroblast cultures and were used as described in details for RANKL/OPG expression and in vitro osteoclastogenic studies.

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

All CM were harvested from explant cultures of synovial tissues and IFMs, and treated and untreated fibroblasts for 6 to 96 hr, were analyzed by ELISA. CM were harvested, centrifuged, and aliquots stored at –70^{0}C. TNF-α, IL-1β, MCP-1, IL-6, IL-8, TGFβ1 and VEGF were determined by using capture ELISAs from R&D Systems.

3.3.3. Fibroblast isolation, culture conditions, treatments and selection of CM

Fibroblasts were isolated from synovial tissues of normal joints and IFMs as described. Medium from fibroblast cultures was changed twice a week and passaged at ~0.7x10^{6} cell density per 10-cm Petri-dish for experiments. Fibroblast cultures only after 6-7 passages were used for in vitro experiments. Fibroblasts were pretreated with various compounds, and the inhibitory concentrations were determined in preliminary experiments. Actinomycin D (2 μg/ml) was used to block transcriptional events, cyclohexamide (10 μg/ml) to inhibit protein translation and synthesis, brefeldin A (1 μg/ml) to

inhibit the transport of freshly synthetized proteins from the endoplasmic reticulum to Golgi complex, monensin (2μg/ml) to block the release of newly sunthetized proteins from Golgi, and cytochalasin D (0.5 μg/ml) to destabilize the cytoskeleton, thus inhibiting phagocytosis. Fibroblasts were pretereated with these compounds for 6 hr in control media (DMEM with 10% FBS), and then replaced fresh DMEM or CM-IFM with or without Ti particles, also containing the original concentration of the inhibitor.

3.3.4. RNA isolation and RNase protection assay (RPA)

Fresh tissue samples (~0.2-0.4 g), and those cultured for 7 days were homogenized with a polytron homogenizer on ice. RNA was extracted with TRIzol as described. TRIzol was also used to isolate total RNA from cultured fibroblasts before and after treatments. RPA was performed on 8 μg of RNA using the Riboquant Multiprobe RNase Protection Assay System according to the manufacturer's directions. After preselecting which commercially available cytokine, chemokine, and growth factor templates can be used, a total of fiveadditional custom-made RPA templates were purchased from BD Pharmingen/Bioscience. The custom-made template #65120 was designated to determine a set of angiogenic factors such as RANTES, IP-10, COX-1, COX-2, bFGF, FGF-R, IL-8, Angioprotein-1, VEGF and c-myc. Template #65238 represented probes for IL-12, GM-CSF-Rα, aFGF, IL-6Rα, M-CSF, IL-6, LIF, TIMP-1 and TIMP-2. The #65184 template was designed to quantify the expression levels of human TNF-α, IL-1RI, IL-4, MMP-1, IFN-γ.

3.3.5. Detection of VEGF isoforms by Western blot hybridization

To detect soluble isoforms of VEGF, the most potent angiogenic factor produced by Ti- and/or CM-IFM-treated fibroblasts, the harvested tissue culture media were loaded on a 10% sodium duodecyl sulfate (SDS)-polyacrylamide gel (PAGE) under reducing conditions. To detect non-secreted, and/or membrane-bound VEGF, treated and untreated cells were lysed in an ice-cold lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% SDS, and 1% NP-40) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 1 unit/ml aprotinin), phosphatase inhibitors (50 mM NaH_2PO_4, 10 mM Na-pyrophosphate, 50 mM KF, and 1 mM Na_3VO_4), and 0.1% NaN_3 for 1 h at 4°C. Cell lysates were cleared by centrifugation and 15 μg of protein per lane was separated by 10% SDS-PAGE in reducing conditions. Proteins were electrophoretically transferred onto nitrocellulose membranes, membranes were blocked with 1% fat-free milk, stained with mAb or rabbit polyclonal antibody to VEGF. Recombinant human VEGFwas used as a positive control, and enhanced chemiluminesence to detect immune reactions.

4. RESULTS

4.1. Examination of RANKL and OPG expression and regulation by human synovial fibroblasts (Study I.)

4.1.1. RANKL and OPG expression by normal and rheumatoid human synovial fibroblasts in response to proinflammatory cytokines

Several studies have demonstrated that human synovial fibroblasts express RANKL on the cell surface and secrete both RANKL and OPG into medium in response to the proinflammatory cytokines TNFα, IL-1β, and IL-17. We first conducted a systemic determination of dose-dependent and time-dependent RANKL and OPG expression using 2 independent normal human synovial fibroblast populations and 3 rheumatoid synovial fibroblast populations. The expression of RANKL and OPG genes was quantified by real-time quantitative PCR, and protein concentrations were determined by ELISA in the cultured media of the same cultures. In dose-response experiments, the TNFα effect reached a plateau at a concentration of 5 ng/ml, and the IL-1β effect reached a plateau at a concentration of 1 ng/ml. These cytokine concentrations were then used in time-course experiments. Concentrations of other cytokines were tested in the same manner, and, as described in materials and methods, 5 ng/ml of IL-4, 5 ng/ml of IFNg, and 25 ng/ml of IL-17 were used in all subsequent experiments employing human synovial fibroblasts. Fibroblasts from RA synovium consistently expressed more RANKL and OPG than fibroblasts from normal synovium in response to the same dose of either TNFα or IL-1β. By 72 hours, the expression of RANKL and OPG was at least 2-4-fold higher in RA synovial fibroblasts than in normal synovial fibroblasts in the same experimental condition, but the sRANKL:OPG ratios were the same in untreated and cytokine-treated cells.

4.1.2. Suppression of RANKL and OPG expression by IL-4 and IFNγ in cytokine-activated normal and RA synovial fibroblasts

Th1 (IFNγ), Th2 (IL-4), and Th17 (IL-17) T cell-produced cytokines are critical mediators of bone metabolism in inflamed synovium. Although IFNγ and IL-4 are considered to be antiosteoclastogenic, IL-17 promotes osteoclast differentiation and activation. Both IL-4 and IFNγ significantly suppressed TNFα- and IL-1β-induced sRANKL levels. Thus, both cytokines might indeed exert antiosteoclastogenic effects via the suppression of TNFα- and IL-1β-induced RANKL expression by fibroblasts. IL-4 alone induced OPG secretion, and this effect was synergistic to the TNFα and IL-1β effects. IFNγ alone did not affect OPG expression but significantly suppressed the OPG levels in both TNFα- and IL-1β-stimulated human fibroblast cultures.

In conclusion, Th2-type IL-4 exhibited a strong antiosteoclastogenic effect on both types of synovial fibroblasts by suppressing TNFα- and IL-1β-induced sRANKL while simultaneously increasing OPG secretion, whereas IFNγ antagonized TNFα- and IL-1β-induced OPG secretion. Although combined

treatment with TNFα plus IL-4 or IL-1β plus IL-4 significantly reduced the RANKL: OPG ratios (a critical factor for osteoclastogenesis), the sRANKL:OPG ratios remained the same after treatment with TNFα plus IFNγ or IL-1β plus IFNγ. IL-17 alone induced both sRANKL and OPG expression, but both factors were significantly lower in IL-17-stimulated cultures than in those treated with either TNFα or IL-1β, and only additive effects could be detected with combination treatments. Although the secreted amounts of sRANKL and OPG (normalized to 1 million cells) were consistently significantly higher in RA FLS cultures, the sRANKL:OPG ratios in RA and normal FLS cultures were comparable when the same cytokine concentrations were used.

4.2 Examination of RANKL and OPG expression and regulation by mouse synovial fibroblasts (Study I.)

4.2.1. RANKL and OPG expression by wild-type mouse synovial fibroblasts from normal and arthritic joints

To test whether proinflammatory cytokine-controlled RANKL and OPG regulation is similar in human and murine systems, we used mouse synovial fibroblasts (also after 4-5 passages) isolated from normal (naive) and arthritic (PGIA) knee joints of wild-type and gene-deficient (IFNγ$^{-/-}$ and IL-4$^{-/-}$) BALB/c mice. As shown for human cells, mouse synovial fibroblasts from arthritic joints expressed approximately twice as much sRANKL and 3-4 times as much OPG in response to either TNFα, IL-1β, or IL-17 than fibroblasts from normal mouse knee joints. In contrast to human synovial fibroblasts, mouse synovial fibroblasts secreted significantly more sRANKL ($P < 0.001$) and less OPG ($P < 0.05$) in response to IL-1β treatment compared with TNFα treatment. Thus, IL-1β seems to be a more osteoclastogenic cytokine than TNFα in the mouse system. Among the cytokines tested, IL-17 alone had a minor effect on RANKL and OPG expression in both naive and arthritic wild-type BALB/c fibroblasts, but no additive effects on RANKL or OPG secretion were detected with combination treatments (TNFα plus IL-17 or IL-1β plus IL-17). Therefore, IL-17 appeared to have no additive effect on sRANKL or OPG expression in arthritic wild-type animals, indicating a limited role of IL-17 on RANKL/OPG balance in pathologic conditions.

4.2.2. Cytokine-mediated RANKL and OPG expression in synovial fibroblasts from gene-deficient mice

The overall trend of sRANKL and OPG expression in synovial fibroblasts isolated from naive and arthritic knee joints of gene-deficient animals was the same as that described for wild-type (BALB/c) fibroblasts. Exogenous IL-4 and IFNγ were able to completely counteract the gene deficiency, significantly ($P < 0.001$) suppressing both TNFα- and IL-1β-induced sRANKL secretion, especially in fibroblast cultures derived from arthritic mouse joints. Compared with IFNγ, IL-4 had a more potent

effect in increasing OPG levels in both TNFα- and IL-1β-stimulated fibroblast cultures. The major difference was that fibroblasts from gene-deficient mice (either IL-4$^{-/-}$ or IFNγ$^{-/-}$) produced 2-4 times more RANKL and 3-5 times less OPG than those from wild-type naive or arthritic mice in the same experimental conditions. Clearly, the sRANKL:OPG ratios were dramatically increased, as much as 1-2 orders of magnitude, in IL-4- or IFNγ gene-deficient mice. IL-17 had a synergistic effect on sRANKL expression, when used in combination with either TNFα or IL-1β, and suppressed or completely blocked OPG secretion by gene-deficient fibroblasts.

4.2.3. RANKL and OPG regulation in wild-type and gene-deficient arthritic mice

The highly coordinated expression of sRANKL and OPG, as reflected by a constant RANKL:OPG ratio in both normal and arthritic synovial fibroblast cultures (either treated or untreated) was completely abrogated in gene-deficient synovial fibroblasts, especially in those exposed to treatment with a combination of proinflammatory cytokines, indicating additional regulatory mechanisms that may exist in vivo. Because RA and its corresponding animal models are all considered to be autoimmune diseases in which the Th1/Th2-type cytokine balance is skewed toward Th1 dominance, wild-type as well as IL-4$^{-/-}$, IL-17$^{-/-}$, and IFNγ$^{-/-}$ mice were immunized with cartilage PG for arthritis induction.

Neither cytokines nor sRANKL were detected in sera from naive mice, and IFNγ, IL-4, and IL-17 were absent in sera obtained from corresponding gene-deficient mice. Levels of all measured serum cytokines (TNFα, IL-1β, IL-6, IL-4, IFNγ, and IL-17) were high in wild-type mice with PGIA but were decreased (by >50%) in IL-4$^{-/-}$ mice, and all cytokine levels were very low, or not detectable, in PG-immunized IFNγ$^{-/-}$ mice. The serum level of IL-17 was barely detectable in arthritic wild-type mice. Serum levels of sRANKL (both "free" and in complex with OPG) were particularly high in IL-4$^{-/-}$ mice and exceeded the serum sRANKL levels measured in wild-type arthritic or IL-17$^{-/-}$ BALB/c mice. In contrast to sRANKL, serum OPG levels were slightly, almost uniformly, elevated in wild-type and all gene-deficient mice with arthritis as compared with naive mice. OPG levels in arthritic wild-type BALB/c mice were comparable with those in nonimmunized IL-4$^{-/-}$ mice, whereas OPG concentrations were lower in IFNγ$^{-/-}$ naive and arthritic mice when compared with those in wild-type mice. In sum, although serum levels of RANKL were high in all PG-immunized mice, and sufficient amounts of OPG were present, only limited amounts of sRANKL were "neutralized" with OPG, except in IL-4$^{-/-}$ mice. This phenomenon was clearly seen in paw extracts, where the RANKL:OPG ratio was the highest in IL-4$^{-/-}$ mice.A more severe arthritis developed in IL-4$^{-/-}$ BALB/c mice, with a significantly earlier onset accompanied by massive bone erosions. A milder form of arthritis developed in IFNγ$^{-/-}$ mice, even after the third PG injection, compared with the severity of PGIA in IL-17-deficient BALB/c mice.

4.3 Examination of angiogenic factors expressed by human synovial fibroblasts (Study II.)

4.3.1. Steady-state mRNA levels in IFM and synovial tissues and selection of "angiogenic" 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 (IFM) soft tissues. For this purpose, we used commercially available Riboquant Multiprobe RPA templates. After the pre-screening of upregulated genes on different RPA templates, three custom-made templates were designed to measure altered gene expressions in both fresh and explant culture tissues, and then in fibroblast cultures. In addition to TNFα, IL-1β, IL-6, IL-8, VEGF and MCP-1, also measured in an earlier study, four more compounds were quantified in both normal synovial tissues and synovial samples from rheumatoid joints or IFM tissues.

4.3.2. Fibroblasts produce angiogenic factors in response to Ti particles, cytokines, chemokines and growth factors

We have shown that fibroblasts phagocytozed particles either in vivo or in vitro, and responded to Ti, inflammatory cytokines, and CM-IFM stimulation. The response was measured at both transcriptional and translational levels. Therefore, we were interested in (i) how the Ti- and/or CM-induced gene expressions correlate, (ii) what is the level, and time frame, of Ti- and CM-induced gene expressions, and (iii) which genes coding for the most relevant angiogenic factors and/or bone resorbing agents are significantly affected by either Ti or CM stimulation. Among the genes differentially expressed in CM-IFM-treated versus untreated cultures, MCP-1 and IL-6, IL-8, b-FGF, a-FGF, TGFβ1, VEGF, Cox-1 and Cox-2 expressions were the most prominent, and were even higher in the combination of CM-IFM plus Ti treatments . In general, the co-treatment had an additive effect upon VEGF, b-FGF and TGFβ gene expression, which was synergistic for IL-8 and Cox-2. Ti alone had no effect on Cox-1 expression in fibroblasts, whereas the CM-IFM induced significant Cox-1 gene expression after 48-hr stimulation. In contrast, both Ti and CM-IFM had an initial, however significant suppressive effect upon Cox-2 gene expression, which then turned to be especially high by 72 hr.

4.3.3. Transcriptional regulation of "angiogenic" factors in fibroblasts in response to stimulation with Ti and/or CM-IFM

As mentioned above, all fibroblasts (from normal or rheumatoid synovial tissue, or from IFM) responded similarly to single (Ti or CM-IFM) or combination treatments. To understand the mechanisms of how and at what level fibroblast activation is affected by either particles, CM, or combination treatments, we used inhibitors to block transcriptional (actinomycin D) or translational (cycloheximide) events, or inhibit intracellular protein transport or phagocytosis via cytoskeleton disorganization. Whenever the IL-6 and VEGF was not secreted (e.g., brefeldin A or monensin-

treatments, data not shown), a negative feed-back pathway suppressed the IL-6- and VEGF-specific mRNA expression as well ($p<0.01$). Other cytokines, chemokines or growth factors exhibited diverse profiles, whereas most of them were regulated at transcriptional, and only marginally at translational levels. The block of intracellular protein transport or secretion did not affect the transcriptional events. Only the TGFβ expression was significantly upregulated in monensin-treated fibroblast cultures, when the TGFβ secretion to culture medium was inhibited.

4.3.4. Transcriptional regulation of VEGF in fibroblasts in response to stimulation with Ti and/or CM-IFM

In a more extended experiment, the expression and secretion of VEGF including the three isoforms were studied using IFM fibroblasts. As expected, the gene expression level of VEGF was time dependent, and CM-IFM with or without Ti particles had the highest response. Most of the VEGF protein expressed in response to CM-IFM was the 55kDa (189 amino acid-long) isoform, but it could be retrieved only in cell lysates, i.e., it was cell surface-, most likely heparan sulphate-bound.

5. DISCUSSION

5.1. Synovial fibroblasts producing osteoclastogenic factors by a cytokine controlled manner (Study I.)

In this study, we have shown that synovial fibroblasts of either human or mouse origin are substantial sources of sRANKL and OPG, and that the production of these mediators is regulated by various cytokines such as TNFα, IL-1β, IL-17, IL-4, and IFNγ. Importantly, proinflammatory cytokine effects were found to be highly comparable in synovial fibroblasts of different origin. Proinflammatory cytokines (TNFα, IL-1 β, and IL-17) consistently increased RANKL mRNA and protein expression in both human and mouse synovial fibroblasts, eventually producing the same levels as those measured in human primary osteoblast and mouse spleen T cell cultures. However, this cytokine-induced sRANKL expression correlated closely with elevated OPG expression. These findings suggest that RANKL production is mostly cytokine regulated and not cell-specific, although different cell types respond differently to cytokine stimulation. In contrast to the large number of factors that are involved in osteoclastogenesis, the bone-protective or bone-resorptive antagonist repertoire of various cytokines and growth factors is limited. In this study, we tested IL-4 and IFNγ to confirm their ability to inhibit osteoclastogenesis and to gain an understanding of how these cytokines affect RANKL/OPG production both in vitro by cytokine-stimulated human synovial fibroblasts and in gene-deficient mice during the progression of inflammatory joint destruction.

Previous studies have shown that IL-4 selectively inhibits TNF signaling, acting directly on both osteoclast precursor cells and mature osteoclasts, and reversibly inhibits osteoclastogenesis through the

inhibition of NF-κB and JNK activation in a STAT-6-dependent manner. IL-4 inhibits RANKL expression by synovial fibroblasts and simultaneously increases OPG secretion. A dramatic shift in the RANKL:OPG ratio can directly affect the differentiation of osteoclast progenitor cells and also inhibits the expression of T cell surface-associated molecules. In this study, we observed that IL-4, alone or in combination with other proinflammatory cytokines, suppressed RANKL production and simultaneously increased OPG expression by fibroblasts. To further confirm this novel observation, we used gene-deficient mouse synovial fibroblasts and observed that RANKL gene expression was highly up-regulated in the absence of IL-4, while OPG production was reduced. Thus, the overall RANKL:OPG ratio became significantly elevated as compared with that in wild-type cells. This elevation was also demonstrated in inflamed joint (paw) extracts from wild-type versus gene-deficient mice with PGIA. This may well explain why we observed unusually aggressive bone resorption in IL-4-deficient mice, supporting the hypothesis that IL-4 is one of the most potent antiosteoclastogenic factors involved in local bone resorption.

Although IFNγ also suppressed proinflammatory cytokine-induced RANKL gene and protein expression, this Th1-type cytokine did not affect, or may even have reduced, OPG secretion in the presence of proinflammatory cytokines. These results, at both the gene and protein levels, were consistent in several independent experiments using normal and RA human synovial fibroblast cultures and IFNγ$^{-/-}$ mice with PGIA. Therefore, these findings, at least in in vitro conditions, appear to contradict the concept that IFNγ has a strong antiosteoclastogenic effect. Although different proinflammatory cytokines seemingly demonstrate strong osteoclastogenic effects via the up-regulation of RANKL, these effects are counteracted by elevated expression of OPG, and most of the released sRANKL is in complex with OPG. However, the osteoclastogenesis-promoting effects of TNFα and IL-1β and the similar, but slightly less prominent, effect of IL-17 are highly regulated by the antiinflammatory cytokine IL-4, more extensively than by IFNγ or any other cytokines tested to date.

In conclusion, it appears that synovial fibroblasts are highly activated cells in the inflamed synovium, and that their cytokine-rich milieu raises the possibility of robust RANKL/OPG production in vivo. The expression of RANKL and OPG is highly regulated by proinflammatory and antiinflammatory cytokines, indicating that synovial fibroblasts may play a substantial role in the initiation and maintenance of bone resorption in inflamed joints.

5.2. Synovial fibroblast plays an important role in angiogenesis and neovascularization (Study II.)

In the first experimental setup of this part of our second study, we have collected synovial tissues from normal, rheumatoid and osteoarthritic joints, and pseudo-(interfacial) membranes of osteolytic lesions to measure the gene expression profiles in fresh tissues and explant cultures, inflammatory cytokines and growth factors in culture media (CM), and the fibroblast responses to various cytokines, chemokines and growth factors detected in CM of explant cultures. Eventually, the periprosthetic microenvironment is very similar to the rheumatoid synovium "supplemented" with an even more drastic local environmental factor: the periprosthetic space is continuously launched with newly generated, non-degradable particulate wear debris. We have shown the overexpression of several angiogenic and osteoclastogenic factors by human IFM fibroblasts (VEGF, MCP-1, M-CSF, IL-8, Cox-1, Cox-2, a-FGF, b-FGF, LIF-1, RANKL and OPG) in response to particulate wear debris and/or cytokine (CM-IFM) stimulation. Cells of this periprosthetic soft tissue, including fibroblasts, are under strong activation pressure due to the continuously generated particulate wear debris, which maintains a chronic state of inflammation. Fibroblasts are actively involved in this detrimental process in that (i) they are continuously stimulated by both prosthetic wear debris and cytokines/growth factors produced by activated macrophages, osteoblasts, and fibroblast (self)-secreted products, (ii) they suppress osteoblast functions, and (iii) they directly or indirectly contribute to osteoclast activation.

While fibroblasts produce large amounts of VEGF in response to various stimuli, they do not respond to VEGF stimulation due to the lack of VEGF receptors Flt-1 and KDR/Flk-1. Exogenous TNF-α significantly upregulated IL-1β, IL-6, M-CSF, MCP-1 and RANKL, and VEGF.

Taken together, macrophage and fibroblast activations are "natural" processes in the IFM, and the effect of fibroblast activation upon angiogenesis and osteoclastogenesis may be as potent and critical as macrophage activation. In addition, activated fibroblasts produce large amounts of bone-resorbing metalloproteinases accompanied by reduced secretion of tissue-specific metalloproteinase inhibitor, which together with a fibroblast-induced suppression of osteoblast function, suggests a significant role for fibroblasts and fibroblast-derived factors in the development of periprosthetic osteolysis.

6. NOVEL FINDINGS:

» Expression of osteoclastogenic factors by human and mouse synovial fibroblasts (Study I.)

- In our first experimental study we demonstrated that synovial fibroblasts of either human or mouse origin are substantial sources of sRANKL and OPG
- The expression of sRANKL and OPG by synovial fibroblasts is highly regulated by proinflammatory and antiinflammatory cytokines
- sRANKL production is mostly cytokine regulated and not cell-specific
- The expression of sRANKL is closly correlated with elevated OPG expression
- We observed that IL-4, alone or in combination with other proinflammatory cytokines, suppressed RANKL production and simultaneously increased OPG expression by fibroblasts. To further confirm this novel observation, we used gene-deficient mouse synovial fibroblasts and observed that RANKL gene expression was highly up-regulated in the absence of IL-4, while OPG production was reduced. Thus, the overall RANKL:OPG ratio became significantly elevated as compared with that in wild-type cells. This elevation was also demonstrated in inflamed joint (paw) extracts from wild-type versus gene-deficient mice with PGIA. This may well explain why we observed unusually aggressive bone resorption in IL-4-deficient mice, supporting the hypothesis that IL-4 is one of the most potent antiosteoclastogenic factors involved in local bone resorption.
- We contradicted the concept that IFN-γ has a strong antiosteoclastogenic effect because IFN-γ also suppressed proinflammatory cytokine-induced RANKL gene and protein expression, but this Th1-type cytokine did not affect, or may even have reduced, OPG secretion in the presence of proinflammatory cytokines.

» Expression of angiogenic factors by human synovial fibroblasts (Study II.)

- In our second experimental study we have shown the overexpression of several angiogenic and osteoclastogenic factors by human IFM fibroblasts (VEGF, MCP-1, M-CSF, IL-8, Cox-1, Cox-2, a-FGF, b-FGF, LIF-1, RANKL and OPG) in response to particulate wear debris and/or cytokine (CM-IFM) stimulation
- Cells of the IFM or rheumatoid synovium produced significantly more bioreactive compounds *in vitro* than those obtained from normal synovial tissues.
- Reciprocally, while fibroblasts produce large amounts of VEGF in response to various stimuli, they do not respond to VEGF stimulation due to the lack of VEGF receptors Flt-1 and KDR/Flk-1
- We found that MCP-1 was as good a marker of fibroblast activation as IL-6, and both secreted compounds (MCP-1 and IL-6) have an effect on osteoclast activation, although this effect is indirect.
- Fibroblasts, via their TNFRp55, might be involved in both RANKL-dependent osteoclastogenesis,[41] and via their TNFα, IL-1 and growth factor receptors in the neovascularization of the IFM.
- Therefore, many of the angiogenic and osteoclastogenic factors detected in the IFM might derive from activated fibroblasts. These fibroblasts and macrophages are present and adjacent to osteoclasts, and because activated fibroblasts secrete RANKL, VEGF and M-CSF, it may well be that the fibroblast is a key cell-type moderating simultaneously both angiogenesis and osteoclastogenesis in the periprosthetic space.

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