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

Miklós Tunyogi-Csapó, MD.

Institute of Musculoskeletal Surgery
Department of Orthopedic Surgery
University of Pécs, Clinical Center
Pécs, Hungary

General President of Post Gradual Education: Sámuel Komoly, MD, PhD, DSc
Program Director: Tamás Illés, MD, PhD, DSc
Mentors: Tibor T Glant, MD, PhD, DSc
Csaba Vermes, MD, PhD
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abbreviations</td>
<td>4</td>
</tr>
<tr>
<td><strong>1. INTRODUCTION</strong></td>
<td></td>
</tr>
<tr>
<td>1.1. Background</td>
<td>5</td>
</tr>
<tr>
<td>1.2. Bone resorption</td>
<td>5</td>
</tr>
<tr>
<td>1.3. Osteoclasts, osteoclastogenesis</td>
<td>6</td>
</tr>
<tr>
<td>1.4. Angiogenesis and hypervascularization in inflamed tissues</td>
<td>8</td>
</tr>
<tr>
<td>1.5. Rheumatoid arthritis and its experimental animal models</td>
<td>10</td>
</tr>
<tr>
<td>1.5.1. Antigen-induced arthritis (AIA)</td>
<td>11</td>
</tr>
<tr>
<td>1.5.2. Collagen-induced arthritis (CIA)</td>
<td>11</td>
</tr>
<tr>
<td>1.5.3. Proteoglycan-induced arthritis (PGIA)</td>
<td>13</td>
</tr>
<tr>
<td>1.6. Pathomechanism of bone resorption in RA</td>
<td>15</td>
</tr>
<tr>
<td>1.7. Potential role of fibroblasts in aseptic prosthesis loosening</td>
<td>17</td>
</tr>
<tr>
<td><strong>2. AIMS AND HYPOTHESIS</strong></td>
<td></td>
</tr>
<tr>
<td><strong>3. MATERIALS AND METHODS</strong></td>
<td></td>
</tr>
<tr>
<td>3.1. Common materials and methods used in all studies</td>
<td>20</td>
</tr>
<tr>
<td>3.1.1. Chemicals and cytokines</td>
<td>20</td>
</tr>
<tr>
<td>3.1.2. Human synovial tissue samples</td>
<td>20</td>
</tr>
<tr>
<td>3.1.3. Fibroblast isolation, and human synovial cultures</td>
<td>21</td>
</tr>
<tr>
<td>3.1.4. Statistical analysis</td>
<td>22</td>
</tr>
<tr>
<td>3.2. Methods for human and mouse osteoclastogenesis study</td>
<td>22</td>
</tr>
<tr>
<td>3.2.1. Mice, immunization, and mouse fibroblast cultures</td>
<td>22</td>
</tr>
<tr>
<td>3.2.2. Treatment of synovial fibroblasts with cytokines</td>
<td>23</td>
</tr>
<tr>
<td>3.2.3. RNA extraction, cDNA synthesis, and RTq-PCR</td>
<td>24</td>
</tr>
<tr>
<td>3.2.4. RANKL, OPG protein, and cytokine ELISAs</td>
<td>24</td>
</tr>
<tr>
<td>3.3. Methods for angiogenesis study</td>
<td>25</td>
</tr>
<tr>
<td>3.3.1. Explant cultures and conditioned media (CM)</td>
<td>25</td>
</tr>
<tr>
<td>3.3.2. Detection of specific protein products by ELISA</td>
<td>25</td>
</tr>
<tr>
<td>3.3.3. Fibroblast isolation, culture conditions, treatments and selection of CM</td>
<td>26</td>
</tr>
<tr>
<td>3.3.4. RNA isolation and RNase protection assay (RPA)</td>
<td>27</td>
</tr>
<tr>
<td>3.3.5. Detection of VEGF isoforms by Western blot</td>
<td>28</td>
</tr>
</tbody>
</table>
4. RESULTS

4.1. Examination of RANKL and OPG expression and regulation by human synovial fibroblasts

4.1.1. RANKL and OPG expression by normal and rheumatoid human synovial fibroblasts in response to proinflammatory cytokines 29

4.1.2. Suppression of RANKL and OPG expression by IL-4 and IFNγ in cytokine-activated normal and RASFs 31

4.2. Examination of RANKL and OPG expression and regulation by mouse synovial fibroblasts

4.2.1. RANKL and OPG expression by wild-type mouse synovial fibroblasts from normal and arthritic joints 34

4.2.2. Cytokine-mediated RANKL and OPG expression in synovial fibroblasts from gene-deficient mice 34

4.2.3. RANKL and OPG regulation in wild-type and gene-deficient arthritic mice 35

4.3 Examination of angiogenic factors expressed by human SFs

4.3.1. Steady-state mRNA levels in IFM and synovial tissues and selection of “angiogenic” factors 40

4.3.2. Fibroblasts produce angiogenic factors in response to Ti particles, cytokines, chemokines and growth factors 42

4.3.3. Transcriptional regulation of “angiogenic” factors in fibroblasts in response to stimulation with Ti and/or CM-IFM 44

4.3.4. Transcriptional regulation of VEGF in fibroblasts in response to stimulation with Ti and/or CM-IFM 46

5. DISCUSSION

5.1. Synovial fibroblasts producing osteoclastogenic factors by a cytokine controlled manner 48

5.2. Synovial fibroblast plays an important role in angiogenesis 51

6. NOVEL FINDINGS

7. REFERENCES

8. BIBLIOGRAPHY

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

1.1 Background

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 endoprostheses 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-inflammatory cytokines can be detected. During the inflammatory 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, granulomatous tissue formation and neovascularization are simultaneous and overlapping events that cannot be separated. 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 loosening and rheumatoid joint destruction.

1.2. Bone resorption

Bone resorption is a mandatory process involved in both physiological and pathological turnover of bone tissue, which means localized degradation of fully mineralized bone matrix, including removal of both inorganic and organic matrix components. At the moment the only cell known to be capable of doing this is osteoclast. Some other phagocytic cells, such as macrophages, have been suggested to resorb bone, but so far they have only been shown to scratch the surface of bone matrix, not to dissolve deep resorption lacunas as osteoclasts do. This feature gives osteoclasts a major role in modeling and remodeling of bone both in physiological and pathological conditions, such as rheumatoid arthritis or periprosthetic osteolysis. Each bone remodeling cycle leads to a local loss of bone due to a deficiency in the amount of new bone formed by decreased osteoblast activity relative to old bone removed by increased activity of osteoclasts. Researchers in the field of bone biology have, for a long time, sought to understand the mechanisms responsible for the ‘crosstalk’ between osteoblasts and osteoclasts. A major step towards answering this question was provided by the discovery of the RANK-RANKL-OPG pathway in the late nineties, and its critical involvement in the cellular regulation of bone remodeling.

Cytokine-mediated RANK/RANKL/osteoprotegerin (OPG) regulation,
pathologic bone resorption in RA (Figure 1.), and RANKL expression on different cell types such as activated T-cells, macrophages, and osteoblasts have been extensively studied, but we have much less information about the involvement of synovial fibroblasts in the bone remodelling process, although fibroblasts are the dominant cell type in the rheumatoid synovium and the periprosthetic interfacial membrane (IFM). These facts led us to investigate synovial fibroblasts via their involvement in pathologic bone resorption.

**Figure 1.** Mechanisms of bone destruction in arthritis. In rheumatoid arthritis, inflammatory synovium invades and destroys bone, a process which is mediated by osteoclasts. Cells in the synovium include synovial macrophages and fibroblasts in addition to infiltrating CD4+ T cells. Interleukin-17 (IL-17)-secreting T helper cells (TH17 cells) are the only osteoclastogenic TH-cell subset (THOc cells) characterized thus far. TH17 cells do not produce interferon-γ (IFN-γ), which suppresses RANKL signaling, but do secrete relatively large amounts of IL-17, which induces RANKL on synovial fibroblasts. IL-17 also stimulates local inflammation and activates synovial macrophages to secrete pro-inflammatory cytokines such as TNF-α, IL-1, and IL-6. These cytokines activate osteoclastogenesis by either directly acting on osteoclast precursor cells or inducing RANKL on synovial fibroblasts. TH17 cells also express RANKL on the cell membrane, which partly contributes to the enhanced osteoclastogenesis (the dotted line) (Schematic figure is adapted from Nakashima et al. J Clin Immunol (2009) 29:555–567)
1.3. Osteoclasts, osteoclastogenesis

Osteoclasts are the only cells that are known to be capable of resorbing bone (Figure 2). Activated multinucleated osteoclasts are derived from mononuclear precursor cells of the monocyte-macrophage lineage. Mononuclear monocyte-macrophage precursor cells have been identified in various tissues, but bone marrow monocyte-macrophage precursor cells are thought to give rise to most osteoclasts. Osteoclast formation, activation, and resorption are regulated by the ratio of receptor activator of NF-κB ligand (RANKL) to osteoprotegerin (OPG; Figure 1), IL-1 and IL-6, macrophage colony stimulating factor (M-CSF), parathyroid hormone, 1,25-dihydroxyvitamin D, and calcitonin. Resorbing osteoclasts secrete hydrogen ions via H⁺-ATPase proton pumps and chloride channels in their cell membranes into the resorbing compartment to lower the pH within the bone-resorbing compartment to as low as 4.5, which helps mobilize bone mineral. Resorbing osteoclasts secrete tartrate-resistant acid phosphatase, cathepsin K, matrix metalloproteinase 9, and gelatinase from cytoplasmic lysosomes to digest the organic matrix, resulting in formation of saucer-shaped Howship’s lacunae on the surface of trabecular bone (Figure 2) and Haversian canals in cortical bone.

The resorption phase is completed by mononuclear cells after the multinucleated osteoclasts undergo apoptosis. RANKL and macrophage CSF (M-CSF) are two cytokines that are critical for osteoclast formation. Both RANKL and M-CSF are produced mainly by marrow stromal cells and osteoblasts in membrane-bound and soluble forms, and osteoclastogenesis requires the presence of stromal cells.
cells and osteoblasts in bone marrow. RANKL belongs to the TNF superfamily and is critical for osteoclast formation. M-CSF is required for the proliferation, survival, and differentiation of osteoclast precursors, as well as osteoclast survival and cytoskeletal rearrangement required for bone resorption (Figure 3).

![Figure 3. Regulation of osteoclastogenesis by receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG): Colony-stimulating factor-1 (CSF-1) normally stimulates osteoclast recruitment. Two forms of RANKL are produced by osteoblasts and osteoblast precursors to stimulate osteoclast recruitment and activation. The membrane-bound form directly interacts with membrane-bound RANK molecules on adjacent osteoclast precursors. The soluble form is released from osteoblasts or osteoblast precursors to diffuse through the intercellular space and interact with membrane-bound RANK molecules on nearby osteoclast precursors. OPG acts as a decoy receptor to prevent RANKL or sRANKL from interacting with RANK. The ratio between RANKL and OPG produced by osteoblasts and osteoblast precursors controls RANKL-stimulated osteoclastogenesis. (Schematic figure is adapted from Bart Clarke et al. Clin J Am Soc Nephrol 3: S131–S139, 2008)](image)

OPG is a membrane-bound and secreted protein that binds RANKL with high affinity to inhibit its action at the RANK receptor. Bone resorption depends on osteoclast secretion of hydrogen ions and cathepsin K enzyme. $H^+$ ions acidify the resorption compartment beneath osteoclasts to dissolve the mineral component of bone matrix, whereas cathepsin K digests the proteinaceous matrix, which is mostly composed of type I collagen. Osteoclasts bind to bone matrix via integrin receptors in the osteoclast membrane linking to bone matrix peptides. The $\beta_1$ family of integrin receptors in osteoclasts binds to collagen, fibronectin, and laminin, but the main integrin receptor facilitating bone resorption is the $\alpha_v\beta_3$ integrin, which binds to...
osteon and bone sialoprotein\textsuperscript{13}.

Binding of osteoclasts to bone matrix causes them to become polarized, with the bone resorbing surface developing a ruffled border that forms when acidified vesicles that contain matrix metalloproteinases and cathepsin K are transported \textit{via} microtubules to fuse with the membrane. The ruffled border secretes $\text{H}^+$ ions \textit{via} $\text{H}^+$-ATPase and chloride channels and causes exocytosis of cathepsin K and other enzymes in the acidified vesicles\textsuperscript{14}. Upon contact with bone matrix, the fibrillar actin cytoskeleton of the osteoclast organizes into an actin ring, which promotes formation of the sealing zone around the periphery of osteoclast attachment to the matrix. The sealing zone surrounds and isolates the acidified resorption compartment from the surrounding bone surface\textsuperscript{15}. Disruption of either the ruffled border or actin ring blocks bone resorption. Actively resorbing osteoclasts form podosomes, which attach to bone matrix, rather than focal adhesions as formed by most cells. Podosomes are composed of an actin core surrounded by $\alpha\nu\beta\alpha$ integrins and associated cytoskeletal proteins.

\subsection*{1.4 Angiogenesis and hypervascularization in inflamed tissues}

Other crucial process in rheumatoid arthritis and periprosthetic osteolysis is angiogenesis which has been always associated with inflammation and pathologic granulomatous tissue development. In both pathologies the inflamed tissues are hypervascularized, which requires angiogenesis and neovascularization. Angiogenesis is the formation of new capillaries from pre-existing vessels. A number of soluble and cell-bound factors may stimulate neovascularization\textsuperscript{16}. The perpetuation of angiogenesis involving numerous soluble and cell surface-bound mediators has been associated with inflammation. These angiogenic mediators, among others, include growth factors, primarily vascular endothelial growth factor (VEGF) and hypoxia-inducible factors (HIFs), as well as pro-inflammatory cytokines, various chemokines, cell adhesion molecules, proteases and others\textsuperscript{17,18}. A perpetuation of angiogenesis leading to enhanced endothelial surface and perpetuated leucocyte ingress into inflamed tissues has been described in Rheumatoid arthritis (RA), as well as other types of arthritis, connective tissue disorders and aseptic loosening of THA\textsuperscript{19,20}. Thus, increased angiogenesis and defective vasculogenesis have important clinical relevance for RA and periprosthetic osteolysis. In our angiogenesis study we were
focusing on angiogenic factors expressed by IFM fibroblasts compared to RA synovial fibroblasts.

Overall, the pathological processes of osteoclastogenesis, dysregulated bone formation, granulomatosus tissue formation and neovascularization are simultaneous and overlapping events that cannot be separated.

1.5 Rheumatoid arthritis and its experimental animal models

Rheumatoid arthritis is a common chronic inflammatory polyarthritis of worldwide distribution, with a female predominance. Although the cause of RA remains unknown, numbers of evidences are suggestive of an autoimmune etiology. Cell-mediated immune response and autoantibodies to cartilage proteoglycans \(^{21}\) and/or collagen type II \(^{22-24}\) have been detected in RA. These autoimmune reactions to cartilage components are, most likely, a consequence of secondary immune response raised against fragments of macromolecules released by local inflammatory processes. A putative figure of human RA summarizes the hypothetical immune mechanisms involved in this disease (Fig.4).

Experimental animal models of inflammatory arthritis (e.g., adjuvant arthritis, various forms of antigen-induced arthritis, collagen-induced arthritis and proteoglycan-induced arthritis) have provided important advances in understanding possible mechanisms for human disease and in developing therapeutic agents for treatment in human pathology. The most frequently studied animal models are those induced in rats or mice\(^{25}\). Short descriptions of the relevant models of RA are included:

1.5.1. Antigen-induced arthritis (AIA)

AIA is based upon an immune response against a foreign antigen to which the animals are sensitized prior to induction of arthritis. Inflammation is induced by local injection of the antigen into the knee joint of the immunized animals\(^{26}\). The severity of local inflammation and subsequent cartilage damage depend on the level of sensitization against the foreign antigen rather that the type of antigen or animal strain\(^{27,28}\). The inflammation is characterized by T-cell infiltration, synovial cell proliferation and loss of aggrecan due to the activation of various matrix metallo
proteinases (MMPs). These MMPs include stromelysin and aggrecanase, which generate neoepitopes with known sequences of aggrecan (–VDIPEN and –NITEGE). This unique condition allows us to detect early cartilage damage by detecting these neoepitopes using specific antibodies.²⁹

![Figure 4. Immunopathogenesis of rheumatoid arthritis (Schematic figure is adapted with minor modifications from Cotran-Kumar-Collins: Pathologic basis of diseases 6th ed.W.B. Saunders Co. 1998.)]
1.5.2. Collagen-induced arthritis (CIA)

Collagen-induced arthritis can be elicited in susceptible strains of rats\textsuperscript{30}, mice\textsuperscript{31} or in monkeys\textsuperscript{32} by immunization with native type II collagen in complete or incomplete Freund’s adjuvant. CIA is induced in susceptible DBA/1 strain of mice by native heterologous type II collagen\textsuperscript{31}. In early stages of CIA, fibrin deposition occurs and anti-collagen antibodies bind to the joint cartilage and activate complement pathways. Erosive polyarthritis typically develops 10-14 days after the primary immunization or a week after a booster injection given intraperitoneally in FCA on day 21. Autoreactive T cells, as well as B cells, which produce antibodies to type II collagen, play critical roles in disease progression.

1.5.3. Proteoglycan-induced arthritis (PGIA)

Immunization of susceptible mouse strains (BALB/c, C3H/HeJCr) with human cartilage proteoglycan induces progressive polyarthritis\textsuperscript{33-35}. The disease develops in all female BALB/c mice when human fetal or newborn proteoglycans are injected intraperitoneally in complete Freund’s adjuvant\textsuperscript{34}. Although fetal human and calf proteoglycans have many biochemical and immunological similarities, calf proteoglycans treated and administered the same way does not produce arthritis in BALB/c mice\textsuperscript{33,34,36}. Thus one may postulate that the arthritogenic structures are present in particular portions of the core protein.

This mouse model shows many similarities to human rheumatoid arthritis and ankylosing spondylitis as indicated by clinical assessments, such as radiographic analysis and scintigraphic bone scans, and by histopathological studies of diarthrodial joints and spine tissue\textsuperscript{33}. During early phases perivascular concentration of mononuclear cells occurs, followed by strong proliferation of synovial macrophages and fibroblasts. The arthritis starts as a polyarticular synovitis in bilateral, small peripheral joints and becomes progressive with extensive erosion of cartilage and bone within the joint. The initial, clinical symptoms of joint inflammation (swelling and redness) appear after the third or fourth intraperitoneal injection of antigen.

Antibodies against immunizing proteoglycan (human) appear during the second/third week of immunization\textsuperscript{34}. T-cell response to proteoglycan is detectable approximately 5-7 weeks after the start of immunization and along the course of the
disease the humoral and cellular immune responses slowly decline as the disease becomes chronic and less active\textsuperscript{34}. The development of arthritis in genetically susceptible mice is based upon cross reactive immune responses between the immunizing fetal human and mouse self-PGs\textsuperscript{33,34,37}. T cells and autoantibodies react with both native and degraded PGs of mouse cartilage and cross-react with the immunizing human fetal proteoglycan\textsuperscript{37,38}.

When aggrecans were compared from ten different species the chondroitin-sulfate depleted aggrecans from fetal or newborn human and canine cartilages were the only ones able to induce cross-reactive T-cell response to mouse proteoglycan and subsequently arthritis\textsuperscript{34,39}.

The important role of GAG side chains (keratan- and chondroitin-sulfate) has recently been discovered. Keratan sulfate side chains can mask dominant arthritogenic T-cell epitopes and their presence may inhibit either antigen presentation or recognition. In contrast, the depletion of the chondroitin sulfate side chains of cartilage aggrecan increases the immunogenicity of the molecule by generating a number of clustered chondroitin sulfate stubs that provoke strong B-cell response and induce professional antigen presenting cells\textsuperscript{38}.

While the antibody level and T-cell response to mouse aggrecan highly correlate with the onset of arthritis, proteoglycan-immunized\textsuperscript{37} non-arthritic animals may also express humoral and cellular immune response against self antigen (mouse aggrecan). T-cell response is linked to MHC (H-2\textsuperscript{d} in BALB/c and H-2\textsuperscript{k} in C3H mice), but DBA/2 or NZB mice with the same haplotype (H-2\textsuperscript{d}) are resistant to PGIA\textsuperscript{34,40}. Furthermore, F1 hybrids (CDF1) of BALB/c and DBA/2 parents (both carry H-2\textsuperscript{d}) are also resistant to PGIA, and the susceptibility returns to 8-28% in F2 hybrids\textsuperscript{36,40}. Further genome-wide screening is being performed at present to identify arthritis-associated quantitative trait loci in PGIA.

T cells, especially Th cells seem to be critical for arthritis induction\textsuperscript{41,42} and are divided into two major groups based upon the cytokine secretion profile: Th1 and Th2. Th1 cells secrete interleukin (IL)-2, interferon (IFN)-\(\gamma\) and tumor necrosis (TNF)-\(\beta\), while Th2 cells produce IL-4, IL-5, IL-10 and IL-13\textsuperscript{43,44}. Th1-type cytokines dominate in the flare up of inflammation in arthritis, thus the Th1 response is considered as pro-inflammatory, whereas the activation of Th2 subset seems to be anti-inflammatory\textsuperscript{45-47}. While the pathological function of immunoglobulin isotypes in arthritis is unclear, Th1 cells and Th1-type cytokines control IgG2a and Th2 cytokines.
the IgG1 production\textsuperscript{48,49}. A critical balance between Th1 and Th2 responses may control the progression and/or severity of arthritis, as CIA is associated with Th1 dominance and IgG2a isotype switch\textsuperscript{50,51}.

1.6 Pathomechanism of bone resorption in RA

Rheumatoid arthritis (RA) is a chronic, inflammatory autoimmune disease characterized by articular cartilage destruction and massive bone resorption. Synovial fibroblasts (RASFs) in RA are one of the dominant cell types in the terminal layer of the hyperplastic rheumatoid synovium and at the sites of invasion into the adjacent cartilage and bone. In normal individuals, the synovial lining at the border to the joint cavity consists of 1–3 cell layers, predominantly containing SFs and macrophages. In RA, the lining thickness increases to 10–15 cell layers\textsuperscript{4,52,53}. In addition to the increased thickness of the synovial lining, the influx and proliferation of inflammatory cells as well as the increased proliferation and survival of resident cells contributes to synovial hyperplasia. The synovium shows an increased neoangiogenesis within the hyperplastic tissue, facilitating the influx of inflammatory cells\textsuperscript{16}. Synovial hyperplasia also contributes to the attachment of the synovium to the adjacent cartilage and bone\textsuperscript{53-55}. The hyperplastic tissue overgrows the underlying cartilage surface and invades into the bone, leading to joint destruction and extensive bone resorption, which is a major problem in RA. A central cell type of cartilage invasion is the SF, which actively contributes to matrix degradation. A main feature of the RA is the highly osteodestructive process, which leads to three forms of bone loss: i) focal bone loss at the joint margins and in underlying subchondral bone (periarticular osteopenia); ii) localized resorption at the site of synovial attachment to bone (erosions); and iii) generalized osteoporosis involving the axial and appendicular skeleton. Of particular interest is the local bone erosion because this radiographic manifestation reflects underlying disease activity, is a key outcome measure, and is associated with an unfavorable prognosis.

Although T cells in RA joints express RANKL\textsuperscript{56,57}, and these RANKL-positive T cells may come in contact with osteoclast precursors\textsuperscript{58}, their direct in vivo osteoclastogenic potential is probably limited. In contrast, T cells in RA joints have a high capacity to produce proinflammatory cytokines that stimulate the expression of RANKL by fibroblast-like synoviocytes (FLS), which may have direct contact with
osteoclast progenitor (macrophage-like) cells. Therefore, T cell-produced cytokines may have an even more important role in osteoclastogenesis than RANKL expression by T cells in the RA joint. However, it is not known how the combination of T cell-derived cytokines can control osteoclast differentiation and how T cells contribute to the extreme bone loss that characterizes the chronic phase of RA. These questions are particularly important for understanding the complexity of RA-associated joint destruction: activated T cells secrete the proinflammatory cytokines tumor necrosis factor α (TNFα), interleukin-1β (IL-1 β), and IL-17, which may induce osteoclastogenesis and osteoclast activation and simultaneously produce antiosteoclastogenic factors such as interferon-γ (IFN γ), IL-4, and IL-10.

IFN γ strongly inhibits osteoclastogenesis, even at minute concentrations, through ubiquitin/proteosome-mediated degradation of TNF receptor (TNFR)-associated factor. However, although IFN γ is abundantly present during the acute phase of RA, this cytokine is barely detectable in the synovium or synovial fluid in chronic disease. Thus, Th1 cells, which can inhibit osteoclastogenesis through IFN γ production, are probably not involved in bone loss. Cytokines that induce Th1 differentiation, such as IL-12 and IL-18, are also inhibitory to osteoclastogenesis. An antiosteoclastogenic effect of IL-4 and IL-10, 2 classic Th2-type cytokines present in RA synovium, has also been described. IL-17, a proinflammatory cytokine produced by activated Th17 T cells, acts on osteoblasts and fibroblasts. Therefore, T cells act directly or through the cytokines they produce, which may have either a harmful or a protective effect in different disease conditions.

Cytokine-mediated RANK/RANKL/osteoprotegerin (OPG) regulation, pathologic bone resorption in RA, and RANKL expression on synovial fibroblasts have been extensively studied, generating a large body of information about the possible involvement of activated T cells and synovial fibroblasts in osteoclastogenesis in vitro and in vivo via their RANKL expression (for review, see refs. and ). In vivo experiments using mice deficient in RANKL, OPG, and a few other factors e.g., NF-κB, TNFα, TNFR, IL-4, IFNγ have clearly documented the critical role of these mediators in bone homeostasis in vivo. However, T cells are rarely seen at the site of bone resorption, either in RA or in corresponding animal models; rather fibroblast-like and macrophage-like cells with
osteoclasts, and less frequently osteoblasts, occupy the resorbed areas of bone.

To gain more insight into the mechanisms of the pathologic bone resorption that takes place in arthritic joints, and to understand how synovial fibroblasts in a cytokine-rich environment are involved in this process, we performed in vitro and in vivo experiments using human and mouse synovial fibroblasts and different cytokine milieus. We compared in vitro expression of osteoclastogenic factors in normal versus RA synovial fibroblasts, then repeated experiments 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.

### 1.7. Potential role of SFs in aseptic prosthesis loosening

Aseptic loosening of joint implants is a disabling condition that can affect patients several years after joint replacement surgery. Total joint replacement for end-stage joint diseases such as osteoarthritis and rheumatoid arthritis is a remarkably successful surgical procedure. Unfortunately, wear debris, from prosthetic components, remains the major factor limiting the survival of joint implants. Periprosthetic osteolysis, due to the formation of an aggressive granulomatous tissue at the bone/cement or bone/ prosthesis interface, is the major clinical problem that leads to the loosening of total joint arthroplasties. Particulate wear debris are continuously generated, phagocytozed, and particulate phagocytosis activates cells of this granulomatous pseudomembrane-like interfacial membrane (IFM). These activated cells then proliferate and/or produce inflammatory mediators, which affect the function of eventually all cell types in either an autocrine or paracrine manner.

Like in the rheumatoid synovium the fibroblast (IFFb) is the dominant cell type in the periprosthetic granulomatous tissue, the interfacial membrane (IFM). Macrophages are the key players in this irresistible process, but other cell types, such as activated fibroblasts, or osteoblast with acquired dysfunction in type I
collagen synthesis\textsuperscript{79,87} are significant contributors to the unbalanced bone remodeling leading to periprosthetic osteolysis and loosing of prosthetic compounds.

We have isolated synovial tissues from normal, rheumatoid and osteoarthritic joints, and compared their gene expression profiles and cytokine/chemokine expressions with those derived from IFM\textsuperscript{87}. Moreover, fibroblasts from all of these tissues were isolated and tested in response to conditioned media (CM) harvested from explant cultures of normal and pathological tissues, and a select group of inflammatory mediators. The most dominant compounds measured in CM of IFM (CM-IFM) were tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)), monocyte/macrophage chemoattractant protein-1 (MCP-1), interleukin-1\(\beta\) (IL-1\(\beta\)), 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-1\(\beta\), IL-6, IL-8, cyclooxygenases (Cox-1 and Cox-2), leukemia inhibitory factor-1 (LIF-1), transforming growth factor \(\beta\)-1 (TGF-\(\beta\)1) and TGF\(\beta\) receptor-I. In addition, IFM fibroblasts expressed RANKL (receptor of activated nuclear factor-kappa B ligand) and OPG (osteoprotegerin) in response to CM-, TNF\(\alpha\)-, or IL-1\(\beta\)-stimulation. Moreover, particulate titanium (used as a “prototype” particulate wear debris) stimulated fibroblasts to express RANKL and these RANKL\(^{+}\) fibroblasts co-cultured with bone marrow cells induced osteoclastogenesis\textsuperscript{97}.

Many components listed above, however, have strong effects on tissue remodeling which, in turn, require angiogenesis and neovascularization. Indeed, the most granulomatous areas of the IFM, especially those resembling rheumatoid synovial tissue, are hypervascularized\textsuperscript{74,77,82,83}. Overall, the pathological processes of osteoclastogenesis, unbalanced bone formation and granulomatous tissue formation occur simultaneously, and overlapping events might not be separated.
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 interface, 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 compounds 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 IFN-γ 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. Common materials and methods in both osteoclastogenesis and angiogenesis studies (Study I. and II.)

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 TNFα, IL-1β, IFNγ, IL-17, and IL-4 were purchased from R&D Systems (Minneapolis, MN) or Sigma. Commercially pure, endotoxin-free small-sized titanium (Ti) particles (<3 μm; Johnson Matthey, Danvers, MA) were characterized earlier\(^{14}\) and used in this study. Based upon the size distribution, a 0.075% Ti suspension (volume/volume: v/v) contained approximately 6x10\(^7\) particles/ml.

3.1.2 **Human synovial 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. Response to bioactive compounds released by cells of the interface membrane, synovial samples from normal joints (negative control), rheumatoid joints and from the interface membrane (IFM) were collected and tested in preliminary and comparative
experiments in both osteoclastogenic and angiogenic studies. 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]).

3.1.3 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 (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) 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). Thus, these cells were considered to be synovial (normal, interfacial membrane or rheumatoid) fibroblasts (i.e., FLS).

Freshly isolated fibroblasts from interface membranes 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 (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. In vitro cultured fibroblasts phagocytosed a substantial number of Ti particles.

3.1.4 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 (Frederick, MD). IL-4−/− and IFNy−/−
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)88. 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, as previously described89. Severe arthritis developed 7-10 days after the second PG injection in all IL-4−/− mice90 and in many of the wild-type89 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 injection91. Arthritis ultimately developed in all wild-type and gene-deficient mice, and the degree of inflammation was assessed visually, as previously described89. The knee joints of age-matched naive (nonimmunized) and PG-immunized wild-type and gene-deficient mice were used for synovial fibroblast isolation, as previously described for human cultures92. 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. Front and hind paws (for which the time of arthritis onset and the inflammation score were recorded) were harvested, homogenized in phosphate buffered saline, and extracted for 24 hours at 4°C.

3.2.2. Treatment of synovial fibroblasts with cytokines.

Confluent cultures of fibroblasts (~1.2-1.5 × 10⁶ cells in a 100-mm petri dish, or 0.6-0.8 × 10⁴ cells/well in a 24-well plate) 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 IFN-γ.
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 (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. The RNA was quantified with a RiboGreen Quantitation Kit (Molecular Probes, Eugene, OR), 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 (Applied Biosystems, Foster City, CA). Serial dilutions ranging from 1:1 to 1:8 of cDNA were amplified using GeneAmp Fast PCR Master Mix (Applied Biosystems). The housekeeping gene GAPDH was used as a reference in each sample. The relative expression of target messenger RNA (mRNA) was calculated from the target threshold cycle (C\textsubscript{t}) values and GAPDH C\textsubscript{t} values, using the standard formula, as described previously\textsuperscript{87}.

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, TNF\textalpha, IL-1\beta, IL-6, IL-17, IL-4, and IFN\gamma 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 (Mountain View, CA). 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 (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. 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°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°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$^{86}$.

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°C. TNF-α, IL-1β, MCP-1, IL-6, IL-8, TGFβ1 and VEGF were determined by using capture ELISAs from R&D Systems (Minneapolis, MN).
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\textsuperscript{84-86,93}. Medium from fibroblast cultures was changed twice a week and passaged at \(~0.7\times10^6\) cell density per 10-cm Petri-dish for experiments. Although cells of the monocyte/macrophage lineage (CD11b\textsuperscript{+} cells) could not be detected after three-four passages with flow cytometry, and over 95% of cultured cells were CD90\textsuperscript{+} (both antibodies from BD Pharmingen/Bioscience, San Diego, CA), confluent monolayer fibroblast cultures only after 6-7 passages were used for in vitro experiments \textsuperscript{86}. Confluent fibroblast cultures in DMEM containing 5% FBS were pretreated with various inhibitors (described later), Ti particles and/or CM-IFM, or various cytokines and growth factors. Reference material was a 5% FBS-containing DMEM (medium control; pre-incubated at 37° for 48 hr) without cytokines and growth factors, and synovial fibroblasts isolated form normal knee joints. Culture media were collected from all particle/cytokine-stimulated and non-stimulated fibroblast cultures at various time points (from 0 hr to 72 hr).

Prior to the experiments of fibroblast stimulation, CM-IFM were individually pretested for cytokine production using a normal synovial fibroblast cell line in 48 hr cultures\textsuperscript{87}. The major selection criteria were that the CM-IFM 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 upon the response (cytokine production) of this normal synovial fibroblast cell line, a total of 16 CM from 21 explant cultures of IFMs (Fig. 1B) were selected and pooled in four groups, each containing 2-5 CM-IFMs\textsuperscript{87}. This was necessary to have a sufficient volume of CM-IFM to test their effect on at least two, usually 3-4, independent IFM fibroblast cell lines (with and without Ti particles), and the results are summarized in this and a previous study\textsuperscript{87}. To answer the question of whether the source of donor tissue (IFM) may affect fibroblast response, each pool of the CM-IFM was prepared so that the IFMs were derived from patients who underwent TJA either due to rheumatoid arthritis (1 group) or osteoarthritis (3 groups), and the primary TJA was performed 6-9 years prior to the revision surgery. The pooled, undiluted CM-IFM contained 20-25 ng/ml TNF-α, 6.5-8.0 pg/ml IL-β, 12-18 ng/ml IL-6, 28-40 ng/ml IL-8, 20-25 ng/ml VEGF, 35-60 ng/ml MCP-1, 0.9-1.2 pg/ml TGFβ and 8-16 ng/ml a-FGF. Technically, there were no significant differences among the effects of these four CM-IFM pools
tested on either normal synovial or IFM fibroblasts, and cumulative results are shown.

Fibroblasts were also 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 synthetized proteins from Golgi, and cytochalasin D (0.5 µg/ml) to destabilize the cytoskeleton, thus inhibiting phagocytosis. Fibroblasts were pretreated 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 (Fig. 1B) were homogenized with a polytron homogenizer (KRI Works, Cincinnati, OH) on ice. RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) as described\textsuperscript{86,87}. 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 (BD Pharmingen) according to the manufacturer’s directions. After preselecting which commercially available cytokine, chemokine, and growth factor templates\textsuperscript{87} can be used, a total of five (three shown on Fig.1) additional 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. [Abbreviations are listed in Figure legend 1.] 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. Custom-made templates included housekeeping genes L32 and glyceraldehyde-6-phosphate dehydrogenase (GAPDH), and templates 65120 and 65184 (but also having IL-4 and IFN-γ) were also used in other experiments\textsuperscript{87}. We found a high correlation (88-96%) between the amount of ribosominal RNA and the message levels of both housekeeping genes L32 or GAPDH; thus the expression of each gene was calculated (normalized) for both housekeeping
genes, and the mean values of L32 and GAPDH were used for normalization.

3.3.5. Detection of VEGF isoforms by Western blot hybridization

To detect soluble isoforms of VEGF\textsuperscript{94}, 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 dodecyl 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\textsubscript{2}PO\textsubscript{4}, 10 mM Na-pyrophosphate, 50 mM KF, and 1 mM Na\textsubscript{3}VO\textsubscript{4}), and 0.1% NaN\textsubscript{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 (BioRad, Hercules CA), membranes were blocked with 1% fat-free milk, stained with mAb (clone 70513 from R&D Systems) or rabbit polyclonal antibody (Santa Cruz Biotechnology, San Diego, CA) to VEGF. Recombinant human VEGF (Santa Cruz) was used as a positive control, and enhanced chemiluminesence (Amersham) 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. However, the condition and source of fibroblasts or FLS were variable in different studies, which might have complicated the interpretation of results. Therefore, 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 (Figure 5). 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 (Figure 5). These cytokine concentrations were then used in time-course experiments (Figure 5, lower panel of each quadrant). 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 IFNγ, and 25 ng/ml of IL-17 were used in all subsequent experiments employing human synovial fibroblasts (Figure 6). 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 (Figure 6). 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 (Figure 6), but the sRANKL:OPG ratios were the same in untreated and cytokine-treated cells (Table 1).
Figure 5. Effect of tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) on the expression of RANKL and osteoprotegerin (OPG) mRNA and protein by human synovial fibroblasts (fibroblast-like synoviocytes [FLS]). Normal (N) FLS and rheumatoid arthritis (RA) FLS after 5-6 passages were treated with different doses of proinflammatory cytokines for 48 hours. Concentrations that achieved a „maximum” effect (5 ng/ml of TNFα or 1 ng/ml of IL-1β) were then used in time-course experiments. Gene expression was normalized to GAPDH and expressed as the fold increase relative to the GAPDH level measured in unstimulated (0 hour) control samples. Secreted proteins were measured by enzyme-linked immunosorbent assay in conditioned media at the given dose and time point, at which time the fibroblasts were trypsinized and counted. Values are the mean and SEM results from at least 3 independent experiments (n ≥ 8-12) involving 2 normal FLS and 3 RA FLS cell lines in duplicate wells.

\* = P < 0.05; \** = P < 0.01, versus normal FLS.

sRANKL = soluble RANKL.
Figure 6. Single and combined effects of proinflammatory and antiinflammatory cytokines on sRANKL (A) and OPG (B) protein expression by human RA FLS. Culture media were collected after 48-hour treatment, and levels of sRANKL and OPG were measured by capture enzyme-linked immunosorbent assay. Treatment combinations and concentrations of different cytokines are shown below the columns. Both sRANKL and OPG levels were significantly higher (at least \( P < 0.05 \)) in RA fibroblast (FLS) cultures than those in conditioned media of normal synovial fibroblasts treated with either TNF\( \alpha \) or IL-1\( \beta \) (with or without IL-4 or interferon-\( \gamma \) [IFN\( \gamma \)]). Neither sRANKL nor OPG showed differences (or differences could not be detected) in untreated, IL-4-treated, or IFN\( \gamma \) -treated normal and RA synovial fibroblast cultures. Values are the mean and SEM results from duplicate wells in 2 independent experiments using 2 normal (n = 8) and 3 RA (n = 12) synovial fibroblast lines. *= \( P < 0.05 \). See Figure 5 for other definitions.

4.1.2. Suppression of RANKL and OPG expression by IL-4 and IFN\( \gamma \) in cytokine-activated normal and RA synovial fibroblasts

Th1 (IFN\( \gamma \)), Th2 (IL-4), and Th17 (IL-17) T cell-produced cytokines are critical mediators of bone metabolism in inflamed synovium. Although IFN\( \gamma \) and IL-4 are considered to be antiosteoclastogenic, IL-17 promotes osteoclast differentiation and activation.\(^4,6,6,69,72,97-99\) As summarized in Figure 6A (only ELISA results are shown), both IL-4 and IFN\( \gamma \) significantly suppressed TNF\( \alpha \)- and IL-1\( \beta \)-induced sRANKL levels. Thus, both cytokines might indeed exert antiosteoclastogenic effects via the suppression of TNF\( \alpha \)- and IL-1\( \beta \)-induced RANKL expression by fibroblasts.
IL-4 alone induced OPG secretion, and this effect was synergistic to the TNFα and IL-1β effects (Figure 6B). IFNγ alone did not affect OPG expression but significantly suppressed the OPG levels in both TNFα- and IL-1β-stimulated human fibroblast cultures (Figure 6B).

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 (Table 1). 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 (Figure 6), and only additive effects could be detected with combination treatments (Table 1). 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 (Table 1).
Table 1. Soluble RANKL (sRANKL) and OPG expression and the sRANKL:OPG ratio in response to cytokine treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>sRANKL</th>
<th>OPG</th>
<th>sRANKL:OPG ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Normal SFs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>0.06 ± 0.06</td>
<td>12.58 ± 1.82</td>
<td>0.005 ± 0.005</td>
</tr>
<tr>
<td>TNFα</td>
<td>7.58 ± 1.06</td>
<td>50.37 ± 11.03</td>
<td>0.150 ± 0.047†</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5.09 ± 0.60</td>
<td>58.21 ± 7.36</td>
<td>0.087 ± 0.014†</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.30 ± 0.18</td>
<td>50.90 ± 5.52</td>
<td>0.006 ± 0.004</td>
</tr>
<tr>
<td>IFNγ</td>
<td>0.12 ± 0.07</td>
<td>11.86 ± 4.53</td>
<td>0.010 ± 0.010</td>
</tr>
<tr>
<td>IL-17</td>
<td>1.97 ± 0.44</td>
<td>31.48 ± 7.41</td>
<td>0.062 ± 0.016†</td>
</tr>
<tr>
<td>TNFα + IL-4</td>
<td>2.26 ± 0.50</td>
<td>168.6 ± 15.38</td>
<td>0.013 ± 0.004††</td>
</tr>
<tr>
<td>TNFα + IFNγ</td>
<td>3.03 ± 0.54</td>
<td>21.34 ± 2.99</td>
<td>0.142 ± 0.038</td>
</tr>
<tr>
<td>TNFα + IL-17</td>
<td>11.5 ± 1.69</td>
<td>89.14 ± 8.09</td>
<td>0.129 ± 0.047</td>
</tr>
<tr>
<td>IL-1 β + IL-4</td>
<td>2.98 ± 0.48</td>
<td>167.11 ± 25.76</td>
<td>0.018 ± 0.005§</td>
</tr>
<tr>
<td>IL-1 β + IFNγ</td>
<td>2.55 ± 0.25</td>
<td>52.75 ± 9.0</td>
<td>0.048 ± 0.011§</td>
</tr>
<tr>
<td>IL-1 β + IL-17</td>
<td>10.1 ± 1.21</td>
<td>109.6 ± 19.11</td>
<td>0.092 ± 0.021</td>
</tr>
<tr>
<td><strong>RA SFs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No treatment</td>
<td>0.17 ± 0.14</td>
<td>20.89 ± 3.52</td>
<td>0.008 ± 0.008</td>
</tr>
<tr>
<td>TNFα</td>
<td>12.73 ± 1.82</td>
<td>87.74 ± 6.05</td>
<td>0.145 ± 0.012†</td>
</tr>
<tr>
<td>IL-1 β</td>
<td>10.84 ± 1.64</td>
<td>101.8 ± 5.43</td>
<td>0.107 ± 0.008†</td>
</tr>
<tr>
<td>IL-4</td>
<td>0.51 ± 0.50</td>
<td>60.2 ± 7.32</td>
<td>0.008 ± 0.010</td>
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<tr>
<td>IFNγ</td>
<td>0.37 ± 0.14</td>
<td>18.14 ± 4.29</td>
<td>0.020 ± 0.011§</td>
</tr>
<tr>
<td>IL-17</td>
<td>3.35 ± 1.09</td>
<td>36.67 ± 6.58</td>
<td>0.091 ± 0.015†</td>
</tr>
<tr>
<td>TNFα + IL-4</td>
<td>5.14 ± 0.89</td>
<td>275.9 ± 32.53</td>
<td>0.019 ± 0.005††</td>
</tr>
<tr>
<td>TNFα + IFNγ</td>
<td>5.86 ± 1.04</td>
<td>38.23 ± 5.52</td>
<td>0.153 ± 0.042</td>
</tr>
<tr>
<td>TNFα + IL-17</td>
<td>16.6 ± 2.35</td>
<td>121.55 ± 53.64</td>
<td>0.134 ± 0.036§</td>
</tr>
<tr>
<td>IL-1 β + IL-4</td>
<td>3.73 ± 1.02</td>
<td>295.96 ± 22.52</td>
<td>0.013 ± 0.004§</td>
</tr>
<tr>
<td>IL-1 β + IFNγ</td>
<td>6.22 ± 0.36</td>
<td>95.80 ± 11.62</td>
<td>0.065 ± 0.010§</td>
</tr>
<tr>
<td>IL-1 β + IL-17</td>
<td>15.89 ± 3.93</td>
<td>150.4 ± 44.49</td>
<td>0.106 ± 0.012</td>
</tr>
</tbody>
</table>

Values are the mean SD results from 9 samples. Soluble RANKL and osteoprotegerin (OPG) concentrations (ng/million cells) were measured in unstimulated or cytokine-stimulated conditioned media at 48 hours. Significant differences between cytokine treatments are shown in Figure 6.

SFs = synovial fibroblasts; IFNγ = interferon-γ; RA = rheumatoid arthritis.
† P < 0.01 versus no treatment.
†† P < 0.01 versus tumor necrosis factor α (TNFα) only.
§ P < 0.05 versus interleukin-1 β (IL-1 β) only.
# P < 0.05 versus IL-1 β only.
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 (Figure 5), 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 (Figures 7A and B; compare wild-type naive with wild-type PGIA). 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 (Figures 7A and B). Thus, IL-1β seems to be a more osteoclastogenic cytokine than TNFα in the mouse system (Figures 7A and B). Among the cytokines tested, IL-17 alone had a minor effect on RANKL and OPG expression (at either the gene or the protein level) 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) (Figures 7A and B). Therefore, IL-17 appeared to have no additive effect on sRANKL or OPG expression in arthritic wild-type animals (see wild-type columns in Figure 7B), 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 (Figures 7A and B). 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 (Figure 7B). 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 (Figure 7; compare wild-type and gene-deficient mice). 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 (Figures 7A and B).

4.2.3. \textit{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 (Figures 7A and B), 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\(^{90,91}\), 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 (nonimmunized) mice, and IFNγ, IL-4, and IL-17 were absent in sera obtained from corresponding gene-deficient mice (Figures 8A-F). 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 (Figures 8A-F), 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 (Figure 8F).
Figure 7. Soluble RANKL and OPG levels measured in synovial fibroblast cultures in response to different treatments. Fibroblasts were isolated from naive and arthritic knee joints of wild-type (WT) and gene-deficient mice. Columns and colors represent the expression levels of sRANKL and OPG in wild-type, interferon-γ (IFN-γ)-, and IL-4-deficient mice without arthritis (A) or with proteoglycan-induced arthritis (PGIA) (B). Single and combination treatments are shown between the sRANKL and OPG panels. The color ranges represent the expression levels, where white indicates no expression at all. KO=knockout (see Figure 9 for other definitions).
Figure 8. A-F, Serum levels of various cytokines, soluble RANKL (sRANKL), and osteoprotegerin (OPG) in naive and proteoglycan (PG)-immunized wild-type (WT) and gene-deficient BALB/c mice. Age-matched wild-type, interferon-γ (IFNγ)-deficient, and interleukin-4 (IL-4)-deficient BALB/c mice were immunized with cartilage PG or were not immunized (naive). Mice were scored for arthritis as previously described ([26]) and killed 14 days after the third PG injection. Sera were collected for cytokine assays (n = 12-16), knee joints were harvested for fibroblast isolation, and paws were collected for histologic assessment and/or tissue extraction (for measuring cytokines, sRANKL, and OPG) (see inset in Figure 9A). Serum sRANKL and OPG levels were measured in both free and complex forms. The cross-capture enzyme-linked immunosorbent assays (ELISAs) detected more sRANKL (up to 150% more) and OPG (up to 8-10% more) than conventional capture ELISAs (compare with results shown in Figure 7). Serum cytokine levels were beneath detectable levels in naive mice but frequently were very high in arthritic mice. G and H, Levels of significance between naive and arthritic mice. Solid lines show significant differences between the sRANKL/OPG complex, and broken lines indicate significant differences between serum levels of “free” sRANKL and OPG. Note the differences between the scales used in G and H. ** = P < 0.01. TNFα = tumor necrosis factor α; PGIA = PG-induced arthritis.
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 (Figure 8G) or IL-17⁻/⁻ BALB/c mice (data not shown). In contrast to sRANKL (Figure 8G), serum OPG levels were slightly, almost uniformly, elevated in wild-type and all gene-deficient mice with arthritis (Figure 8H) as compared with naive mice. OPG levels in arthritic wild-type BALB/c mice were comparable with those in nonimmunized (naive) IL-4⁻/⁻ mice, whereas OPG concentrations were lower in IFNγ⁻/⁻ naive and arthritic mice when compared with those in wild-type mice (Figure 8H). In sum, although serum levels of RANKL were high in all PG-immunized mice (Figure 8G), and sufficient amounts of OPG were present, only limited amounts of sRANKL were “neutralized” with OPG, except in IL-4⁻/⁻ mice (Figures 8G and H). This phenomenon was clearly seen in paw extracts, where the RANKL:OPG ratio was the highest in IL-4⁻/⁻ mice (see inset in Figure 9A).

A more severe arthritis developed in IL-4⁻/⁻ BALB/c mice, with a significantly earlier onset accompanied by massive bone erosions (Figures 9D and Dd). A milder form of arthritis developed in IFNγ⁻/⁻ mice, even after the third PG injection (Figure 9C), compared with the severity of PGIA in IL-17-deficient BALB/c mice (Figure 9E).

Although in vivo conditions may differ substantially from in vitro fibroblast cultures, the prominent osteoclast formation adjacent to FLS, particularly in wild-type, IL-4⁻/⁻, and IL-17⁻/⁻ mice (Figures 9Bb, Dd, and Ee), suggests a critical role of fibroblast-derived (either membrane-bound or soluble) RANKL in promoting osteoclastogenesis under inflammatory conditions.
Figure 9. Histologic sections of tarsometatarsal joints from wild-type (WT) and gene-deficient arthritic BALB/c mice, with or without proteoglycan-induced arthritis (PGIA). A, Paws from naive (nonimmunized) mice (either WT or gene deficient) exhibited no histopathologic abnormalities. No cytokines, RANKL, or osteoprotegerin (OPG) was detected in paw extracts from the corresponding framed areas (results not shown). B-E, Representative sections from wild-type and gene-deficient (knockout [KO]) mice with proteoglycan-induced arthritis. Higher-magnification views of boxed areas in B, C, D, and E are shown in Bb, Cc, Dd, and Ee, respectively. The most extensive bone resorption was seen in WT (B) and interleukin-4 (IL-4)-deficient (D) mice (double black arrows). Numerous osteoclasts (multinucleated cells, as indicated by the white arrows) “cover” the resorbed bone surface, and resorption pits are clearly seen in adjacent cortical bone. Tissue destruction and bone resorption were highly comparable in each arthritic mouse, even though the RANKL:OPG ratios were dramatically different in gene-deficient mice (inset in A; values are the mean and SEM results from 4 paws of 5-8 mice). (Hematoxylin and eosin-stained; original magnification × 10 in A-E; × 40 in Bb, Cc, Dd, and Ee.) IFNγ = interferon-γ.
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\(^87\). For this purpose, we used commercially available Riboquant Multiprobe RPA templates (hCK3, hCK4, hCK26, hCR4, hCR5, hCR6 and hAngio-1). After the pre-screening of upregulated genes on different RPA templates, three custom-made templates were designed (Fig. 10) to measure altered gene expressions in both fresh and explant culture tissues (Fig. 11), and then in fibroblast cultures (Fig. 12). In addition to TNF\(\alpha\), IL-1\(\beta\), IL-6, IL-8, VEGF and MCP-1, also measured in an earlier study\(^87\), four more compounds were quantified in both normal synovial tissues and synovial samples from rheumatoid joints or IFM tissues (Fig. 11A). As summarized in Figure 11 and in an earlier experiment\(^86,87\), normal synovial tissues expressed significantly less message for any cytokines, chemokines and growth factors than those measured in either rheumatoid synovial tissue or IFM. The gene-specific mRNA expression continuously increased up to day 7 (at the end of explant cultures), when the message levels for all measured genes were approximately 2-10-fold higher (Fig. 11B) than in fresh samples (Fig. 11A). This was reflected by secreted cytokines and growth factors (data not shown).
Figure 10. The effect of titanium (Ti) particles and conditioned media (CM) of interfacial membranes (CM-IFM) on gene expression by fibroblasts isolated from the IFM. Three custommade templates show representative RPAs using radiolabeled cRNA probes from untreated, Ti- and/or CM-treated fibroblasts of IFM after 48 h treatment. 32P-labeled transcripts of known size were generated by in vitro transcription from RNA templates and were used as size markers. Template 65120 [also used in reference15] contained: RANTES, a CXC chemokine regulated upon activation normally T-cell expressed and secreted; IP-10/CXCL-10 CXC chemokine; Cox-1/2 cyclooxygenase 1 and 2; bFGF basic fibroblast growth factor (FGF-2); FGF-R FGF receptor; Ang-1 angiopoietin 1; VEGF vascular endothelial growth factor; c-myc oncogene. Template 65238: IL-12p40 Interleukin-12p40 subunit, GMCSF-Ra granulocyte-macrophage colony-stimulatory factor receptor-alpha; aFGF acid fibroblast growth factor (FGF-1); IL-6Ra interleukin-6 receptor- alpha; M-CSF macrophage colony-stimulatory factor;LIF leukemia inhibitory factor-1; TIMP-1 and TIMP-2 tissue inhibitor of metalloproteinases-1 and -2. Template 65184-b: TNFa tumor necrosis factor-a; IL-1RI IL-1 receptor-I; IL-1a and IL-1b; MMP-1 matrix metalloproteinase-1 (collagenase); MCP-1 monocyte chemoattractant protein-1; TGF-b1 transforming growth factor-b1; TGF-bRI/TGF-b receptor-I. Dashes indicate the corresponding gene’s position [occasionally two or three transcripts (e.g., VEGF or IL-8) can be seen], whereas the intensities of bands show the level of mRNA expression. Because there were some but inconsistent differences (<15% variance) betweenL32 andGAPDH (glyceraldehyde-6-phosphate-dehydrogenase) housekeeping genes, all gene expressions were normalized to both L32 and GAPDH, and then the mean values of the two independent normalization processes were used, and then data are shown in all subsequent figures. Treatments are indicated underneath the templates. Representative panels of over 12–15 hybridization experiments are shown.
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\(^\text{87}\), and responded to Ti, inflammatory cytokines, and CM-IFM stimulation (Fig. 12). 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. To answer these questions, IFM-fibroblasts were untreated (i.e., cultured in medium control), or treated with CM-IFM without or with 0.075% (v/v) Ti particles for different time periods. The gene expression levels of a select group of angiogenic/osteoclastogenic factors by normal synovial and IFM-fibroblasts treated with Ti particles or CM-IFM, or both, were compared in a time-dependent manner (Fig. 12). Both types of fibroblasts (normal synovial and IFM) exhibited very similar compound- and time-dependent responses for each gene measured, although the IFM fibroblast response was significantly more extensive than those measured in normal synovial fibroblasts (Fig. 12). Although Ti particles in a “plane” DMEM with 5% FBS (medium control) induced a smaller but still significant gene expression, the co-treatment of fibroblasts (either normal or IFM) especially with CM-IFM and Ti dramatically upregulated the expression of all genes studied (Fig. 12, upper segments of each panel). 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 (Fig. 10; last lane on each template shows fibroblast response to this combined treatment). 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 (Fig. 12).
Individual cytokines, chemokines and growth factors (IL-6, MCP-1, IL-8), except TNFα and IL-1β, either alone or in combination with Ti particles, had no or only a marginal, effect upon Cox-1, Cox-2, TGFβ, b-FGF, IL-8 and VEGF gene expressions (data not shown). However, when these cytokines/growth factors were combined in a cytokine “cocktail” (positive control)\(^7\), their effect on gene expression was dramatic and highly comparable with those of CM-IFM (Fig. 12). In general, for selected fibroblast activation markers (Fig. 10), the highest gene expression was achieved between 48- and 72-hr treatments (Fig. 12), whereas the highest amounts of secreted proteins were measured in 72-96 hr culture media\(^7\) (data not shown).

**Figure 11.** Steady-state mRNA levels measured in fresh normal (NSy) and rheumatoid (RASy) synovial tissues and interfacial membranes (IFM) (A), and then after 7-day culture period (B). mRNA levels were measured by RNase protection assay (RPA) normalized to expression of L32 and GAPDH housekeeping genes in the same sample. Numbers (n) of samples are indicated. Numbers (n) of samples are indicated. The expression levels of Cox-1, Cox-2, and basic-FGF were highly comparable in the three different types of tissues, but the TGF-b, IL-8, and VEGF were significantly higher for RASy and IFM compared to NSy in both fresh tissues and explant cultures. Because there were no significant differences in gene expressions between RASy and IFM tissues, and the fibroblast “activationmarkers” (such as IL-6, VEGF, and IL-1b protein contents) were highly comparable, CM-IFM were used in subsequent experiments on two independent IFM fibroblast lines. Gene expression data for IL-8 and VEGF are combined with those obtained in a previous study.\(^15\) Values are shown as mean SEM.
Figure 12. Time-dependent mRNA expression coding for VEGF, TGF-b, basic-FGF (FGF-2), IL-8, Cox-1, and Cox-2 in normal synovial and IFM fibroblasts cultured for 12–72 h in normal DMEM with 5% FBS (reference medium) exposed to Ti particles (0.075%, v/v) and/or CM-IFM treated samples. Gene expression levels in normal synovial fibroblasts were consistently lower (open symbols) than IFM fibroblasts (closed symbols), and the maximum effect was achieved at 72 h experiments in Ti plus CM-IFM treatment (except for TGF-b). Note, different scales are used for Ti versus CM-IFM or CM-IFM/Ti treatments. The mean SEM values are also indicated by the level of significant differences compared to the 12-h values: *p<0.05, **p<0.01, and ***p<0.001.
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. As shown on Figure 13 for IL-6, a prominent cytokine of fibroblast activation, and for VEGF, the most prominent pro-angiogenic factor, the effect of CM-IFM was blocked at transcriptional level (p<0.001). 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 (data not shown). The block of intracellular protein transport or secretion did not affect the transcriptional events (data not shown). Only the TGFβ expression was significantly upregulated in monensin-treated fibroblast cultures, when the TGFβ secretion to culture medium was inhibited (data not shown). Technically, the Ti effect for each measured cytokine, chemokine or growth factor was completely blocked by cytochalasin D, whereas other inhibitory compounds had minor effect upon gene or protein expression in Ti-stimulated fibroblast cultures. In conclusion, treatments of fibroblast with either CM-IFM and/or Ti particles affected the most upstream events, and the expression of fibroblast activation markers were controlled at transcriptional levels.
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 and summarized on Figure 10, the gene expression level of VEGF was time dependent, and CM-IFM with or without Ti particles had the highest response (Fig. 14A). 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. In contrast, the 23 kDa (165 amino acid-long) isoform was secreted.
acid-long) and 18 kDa (121 amino acid long) VEGF isoforms were well detected in the medium. Ti particles had significant effect on the secretion of the 23 kDa VEGF isoform, but eventually no effect was detected for the 18 kDa and 55 kDa isoforms (Fig. 14B). Gene and protein expression profiles of VEGF in response to various treatments are summarized on figures 14C and 14D.

**Figure 14.** Gene expression profile and secretion of VEGF by IFM fibroblasts in response to various treatments. Panel (A) shows the fold-increase of VEGF expression after 12- and 24-h treatments with Ti and/or CM-IFM. Panel (B) has representative Western blots showing the three protein isoforms. The 55 kDa (VEGF189 isoform) was present in a membrane-bound form and could be detected only in cell lysates, whereas the 18 (VEGF121) and 23 kDa (VEGF165, the most dominant and most potent angiogenic isoform detected as a homodimer) splice variants were secreted and measured in 72-h CM of IFM fibroblasts. Treatments are indicated underneath the Western panels. Panel (C) summarizes the gene expression measured by RPA (48-h), and panel (D) the secreted amounts of VEGF measured by ELISA in the same media. Based upon the Western blot results, these measurements represent only the 18- and 23-kDa isoforms. Treatments are indicated underneath the columns, and levels of significant differences (mean SEM) are shown: *p<0.05 and **p<0.01 (nL 3–5 samples).
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 (human and mouse, normal versus arthritic). 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 (our workgroup’s unpublished observations). 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\textsuperscript{100,101}, the bone-protective or bone-resorptive antagonist repertoire of various cytokines and growth factors is limited\textsuperscript{4,52,71,99}. 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.

The binding of RANKL (either soluble or as a cell surface receptor) to RANK expressed on the surface of osteoclast precursor cells is probably the most important event in the induction of osteoclastogenesis\textsuperscript{58}, which can be monitored in in vitro osteoclastogenesis assays in the presence of various proinflammatory cytokines\textsuperscript{100,101}. The decoy receptor OPG competes with RANK-RANKL binding by blocking their interaction\textsuperscript{102-104}. Therefore, the RANKL:OPG ratio appears to be a critical parameter in osteoclastogenesis, controlling bone homeostasis in both normal and pathologic conditions\textsuperscript{4,52,71,99}. Although the majority of RANKL is probably expressed as a cell-
surface receptor in several activated cell types\textsuperscript{4,105,106}, significant amounts of circulating sRANKL (either as a splice variant without transmembrane domain or as a shed form) and OPG are detectable in the circulation\textsuperscript{97,105}. At least one of the splice variants of sRANKL, possibly more in the mouse system (http://www.ensembl.org/Mus_musculus/geneview), is inactive due to the lack of a binding site to OPG as described in the human system\textsuperscript{107}; thus, such variants can be measured as free sRANKL (Figure 8G).

Based on the results of cross-capture ELISAs (Figures 8G and H), one of the anti-osteoclastogenic effects of IL-4 may be explained by the fact that IL-4 does not only suppress RANKL production, but probably a lesser amount of sRANKL can bind to OPG. Although this amount of sRANKL that cannot bind to OPG may be very small relative to the cell surface-bound RANKL\textsuperscript{87}, a correct conclusion can be made only using in vitro osteoclastogenesis assays\textsuperscript{86,100,101}, in which known amounts of free sRANKL and sRANKL/OPG complex are tested. Two of the 3 isoforms of sRANKL appear to be biologically as active as the membrane-bound form\textsuperscript{97,105,108} thus, the sRANKL:OPG ratio may be as relevant a marker of osteoclastogenesis as either membrane-bound RANKL or circulating OPG alone\textsuperscript{105}. The expression levels of RANK, RANKL, and OPG are under the control of various cytokines, but a portion of serum sRANKL and OPG may also be nonskeletal in origin\textsuperscript{4,56,98,106}. Therefore, the serum levels of sRANKL and OPG may not necessarily reflect cytokine activities in the bone microenvironment, whereas circulating sRANKL and OPG may significantly affect osteoclastogenesis in vivo\textsuperscript{109}.

In an inflammatory environment such as that in RA joints, T lymphocytes, macrophages, and fibroblasts are activated and produce numerous cytokines, which in turn further activate surrounding cells in either a paracrine or an autocrine manner. Osteoclast progenitor cells express RANK, whereas the major sources of RANKL (and OPG) are activated T lymphocytes, osteoblasts, and fibroblasts. Therefore, additional studies are necessary (and are in progress) to detect the biologic activities of free, fibroblast-expressed, and OPG-bound sRANKL in in vitro osteoclastogenesis assays (Glant T, et al: unpublished observations). However, in these in vitro assays, whenever we measure the sRANKL:OPG ratio (as shown in Figure 9A), special care and selection of antibodies are necessary to detect the RANKL/OPG interaction site,
which should not block the antibody-binding sites (epitopes) of any of the 2 components.

Both fibroblast-like and macrophage-like cells populate the inflamed RA synovium, especially during the chronic phase of disease, and these cells occupy the space adjacent to the resorbed bone. Moreover, these cells appear to be in contact with each other and frequently are in contact with osteoclasts (Figures 9Bb and Db).

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 (Figure 7). 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 (Figure 9D), 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 (Figure 6). 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.
Conclusion of osteoclastogenesis study:

In conclusion, 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 (Figure 8G). 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. Although these experiments with fibroblasts require additional in vitro and in vivo studies that focus on cytokine/fibroblast-mediated aspects of osteoclastogenesis, our observations suggest that, indeed, activated RANKL-expressing fibroblasts are capable of inducing osteoclast formation in vitro86,87,112. 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 cultures87. A number of cytokines/growth factors with or without Ti wear debris upregulated various factors, including RANKL, OPG, and a group of (pro)angiogenic factors. Although the macrophage/monocyte activation is probably the most prominent event in the periprosthetic environment76,77,82,83,93,113, fibroblasts with high proliferative rate114,115 comprises approximately 30% of the total cell number of the IFM76,82,116. Fibroblasts can also phagocytose particles both in vivo and in vitro87, produce and respond to a
number of inflammatory mediators and growth factors\textsuperscript{84,86,87,115-118}, and last but not least, these cells express RANKL in response to Ti phagocytosis, TNFα or IL-1β stimulation, which level of RANKL expression seems to be sufficient to induce osteoclastogenesis\textsuperscript{86,87,119}. The periprosthetic granulomatous soft tissue (IFM) exhibits a heterogeneous histopathological feature, in which highly vascularized areas with dens cellularity, especially adjacent to the osteolytic lesion, alternate with fibrotic and pseudocapsule-like tissue structures\textsuperscript{74,77,82,83,113,120}. In both cases, the angiogenesis is critical and initiated by cytokines, hypoxia and pro-angiogenic factors to supply necessary nutrition conditions. Thus, the maintenance and nutrition of this heterogeneous but highly dynamic tissue of macrophages, fibroblasts and foreign body giant cells of the periprosthetic milieu requires sufficient blood supply, thus the angiogenesis and neovascularization initiated by a cocktail of growth factors and pro-angiogenic cytokines are crucial to prevent tissue necrosis. On the other hand, these angiogenic factor may act as activators of increased bone resorption. Angiogenesis plays a central role in the pathogenesis of a number of pathological processes, such as in tumor growth, eye diseases, wound healing and chronic inflammatory diseases (e.g., psoriasis or rheumatoid arthritis)\textsuperscript{16,121,122}. 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. In turn, cells of this periprosthetic soft tissue, especially in the osteolytic areas, are constantly activated, which maintain an irresistible and irreversible process.

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\textsuperscript{113,114,117,123}. 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\textsuperscript{84,86,87,93,118,119,124}.
To gain insight into the complex mechanisms taking place in the periprosthetic space, and to understand how these fibroblasts are involved in the formation of IFM and osteolysis, we and others have used fibroblasts from different sources (synovial tissues of normal and pathological joints, and from IFMs) and tested their responsiveness under different conditions\(^{86,87,93,116}\). In this study, we compared the responsiveness of human fibroblasts of different origins to Ti particles and CM harvested from IFMs. While fibroblasts from normal synovial tissues could be stimulated less effectively than those isolated from IFM using the same CM-IFM (Fig. 10), CM from normal synovial tissues had no or minimal effect upon fibroblasts isolated from either normal or pathological tissues (unpublished data). Thus, while the \textit{in vitro} responsiveness of fibroblasts derived from either a normal or inflammatory milieu were highly comparable after a few passages, cells of the IFM (or rheumatoid synovium, data not shown) produced significantly more bioreactive compounds \textit{in vitro} than those obtained from normal synovial tissues.

To mimic \textit{in vivo} conditions of the periprosthetic pathological bone resorption as closely as possible, we stimulated IFM fibroblasts with Ti particles and/or CM from IFMs. In order to reproduce the \textit{in vivo} conditions, particles of approximately the same size distribution as the wear debris present in periprosthetic tissues\(^{78,125-127}\) were used to simulate fibroblasts. In general, fibroblasts from IFMs associated with various degrees of osteolysis responded very similarly after 6-8 passages, but the variability in cytokine/chemokine concentrations in collected CM had a more diverse effect upon fibroblast activation. Therefore, we selected a model system in which we could compare activation events of several fibroblast lines using CM from a number of IFMs with high levels of TNF-\(\alpha\), IL-1\(\beta\), IL-6, IL-8, MCP-1, FGFs and VEGF, factors which are involved in both angiogenesis and osteoclastogenesis.

We found that MCP-1 was as good a marker of fibroblast activation as IL-6\(^{87}\), and both secreted compounds (MCP-1 and IL-6) have an effect on osteoclast activation, although this effect is indirect\(^4\). While fibroblasts express both TNFRp55 and TNFRp75\(^4,95\), and respond to TNF-\(\alpha\) stimulation (Fig. 14C-D), neither synovial nor IFM fibroblasts produce TNF-\(\alpha\) in response to Ti particle or CM-IFM stimulation\(^{87}\). Reciprocally, while fibroblasts produce large amounts of VEGF in response to various stimuli (Fig. 14D), they do not respond to VEGF stimulation due to the lack of VEGF receptors Flt-1 and KDR/Flk-1\(^{94,128}\). Exogenous TNF-\(\alpha\) significantly upregulated IL-1\(\beta\), IL-6, M-CSF, MCP-1 and RANKL\(^{87}\), and VEGF
Thus, fibroblasts, via their TNFRp55, might be involved in both RANKL-dependent osteoclastogenesis$^{129}$, and via their TNFα, IL-1 and growth factor receptors in the neovascularization of the IFM.

Among the fibroblast activation markers induced by either TNF-α, IL-1β, particulate Ti or CM-IFM the expression of membrane-bound 55 kDa VEGF isoform together with soluble forms (23 kDa and lesser amounts of 18 kDa) of VEGF isoforms (all potent pro-angiogenic factors) (Fig. 14) were also detected. In addition, activated fibroblasts secreted Cox-2, LIF-1, IL-8, TGFβ1, a-FGF and b-FGF, all involved in both angiogenesis and osteoclastogenesis$^{4,130-132}$. Therefore, many of the angiogenic and osteoclastogenic factors detected in the IFM$^{82,83,118,133,134}$ might derive from activated fibroblasts (Figs. 10 and 12). These fibroblasts and macrophages are present and adjacent to osteoclasts$^{77}$, 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.

Conclusion of angiogenesis study:

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$^{84}$, which together with a fibroblast-induced suppression of osteoblast function$^{79}$, 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 closely 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 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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