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Role of the Osteoblast in the Development of Periprosthetic Osteolysis and Aseptic Implant Loosening

Csaba Vermes, M.D.

Department of Orthopedic Surgery

Faculty of Medicine, University of Pécs, Hungary

General President of Post Gradual Education: Judit Nagy, M.D., Ph.D., D.Sci.

Program Director: Árpád Bellyei, M.D., Ph.D., D.Sci.

Mentors: Tibor T. Glant, M.D., Ph.D., D.Sci.

György Lovász, M.D., Ph.D.

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LIST OF ABBREVIATIONS USED IN CHAPTER 1 AND CHAPTER 4

AP	Alkaline phosphatase
ATP	Adenozine-triphosphate
BAG-75	Bone acidic glycoprotein-75
BMP	Bone morphogenic protein
Cbfa-1	Core binding factor-1
CD-44	Cluster of differentiation-44
Dlx-5	Distal-less homeobox-containing gene-5
DMP-1	Dentin matrix protein-1
DNA	Dezoxy-ribonucleic-acid
E-11	Osteocyte specific antigen-antibody
FGF	Fibroblast growth factor
Fra-2	member of the activating protein-1 (AP-1) transcription factor family
IGF	Insulin like growth factor
IL	Interleukin
LIF	Leukemia inhibitory factor
MCP	Macrophage chemoattractant protein
Msx-2	Msx-class homeobox (Hox) gene-2
NF-κB	Nuclear factor-kappaB
OB-7.3	Osteocyte cell surface Phex recognizing antibody
PDGF	Platelet-derived growth factor
PDTC	Pyrrolidine dithiocarbamate
PE	Polyethylene
PGE	Prostaglandin E
PTH	Parathyroid hormone
RCC-455	Osteocyte/osteoblast specific antigen-monoclonal antibody
SB-1-10	Osteocyte specific antigens-antibodies
Sca-1	Osteocyte specific antigen-antibody
Stro-1	Osteocyte specific antigen-antibody
TGF	Transforming growth factor
TNF	Tumor necrosis factor

CHAPTER 1

INTRODUCTION

One of the main issues in orthopedic surgery is the treatment of various end stage joint diseases affecting millions of people. Total joint replacement using metallic and polymeric materials has emerged as one of the success stories of modern orthopedic surgery. Inserting artificial materials into the human body, however, produces new problems. Among these problems is the increasing recognition that, in the long term, total joint arthroplasty may be associated with adverse local and remote tissue responses. These adverse effects are mediated by the degradation products of implant materials, which may be present as particulate wear debris, organometallic complexes or metals in ionic forms. The presence of these products ultimately influences the bone turnover and remodeling locally in the periprosthetic area leading to an unbalanced bone metabolism caused by either increased osteoclastic bone resorption or decreased osteoblastic bone formation resulting in less organic bone matrix and mineral deposition. This process generates the clinically well-known periprosthetic osteolysis contributing to aseptic implant loosening and leading eventually to implant failure and subsequently to revision joint surgery.

This section will discuss some basic physiology of the osteoblast, the extracellular bone matrix and bone remodeling, and gives a brief overview of the pathophysiology of periprosthetic osteolysis.

The osteoblast

Bone is a specialized active connective tissue that forms, along with cartilage, the skeletal system. The basic elements are the cells and the extracellular matrix in bone. Bone comprises an exquisite assembly of functionally distinct cell populations that are essential to

support the structural, biochemical, and mechanical integrity of this mineralized tissue and its crucial role in mineral homeostasis. Stromal osteoprogenitor cells contribute to maintaining the populations of osteoblasts, osteocytes and bone-surface lining cells. These cells are the fundamental cells that mediate the bone forming processes of the skeleton. Progenitors of the bone-forming cells are derived from the mesodermal germ cell layer thus they have mesenchymal origin. The periosteum and bone marrow are important sources of mesenchymal progenitor and osteoprogenitor cells. These precursors with the right stimulation undergo extensive proliferation and differentiate into preosteoblasts. Transcription factors and regulatory proteins that directly engage in protein-DNA as well as in protein-protein interactions also are important for the development of bone-tissue and osteoblast differentiation. Some of the cytokines, hormones, growth factors and various transcription factors mediating commitment of stem cells to osteoblast lineage, and influencing osteoprogenitor growth and differentiation, are shown in Table 1.

Developmental Stage	**Surface Markers**	**Phenotype Influencing Factors**	**Transcriptional Factors**
Stem Cell	Sca-1, Stro-1	BMPs, TGF-βs	
Stromal Mesenchymal Cell (inducible osteoprogenitor)	SB-10	LIF, FGFs, PDGF	Cbfa-1, Msx-2,
Osteoprogenitor (determined)	SB-2, 3, 4	PTH, $1,25(OH)_2D_3$, PGE2, IGF-1, IGF-2, TGF-β, Glucocorticoids, Cytokines	
Committed Preosteoblast	E-11, RCC-455, AP, Collagen I and III, Osteopontin		Cbfa1, c-fos
Osteoblast	E-11, SB-2, AP, Collagen I and V, Bone sialoprotein, Osteopontin, Osteocalcin		Fra-2/jun-D, Dlx-5
Osteocyte	SB-3, CD-44, OB-7.3		

Table 1: Regulation of osteoblast differentiation. The first column represents the stage of osteoblast development. The second column shows the cell surface molecules or proteins, which are characteristic for the phenotype. The third and fourth columns indicate the various soluble mediators and transcription factors, respectively, contributing to the phenotype[1-9].

Committed preosteoblasts in a nondividing phase are localized in bone proximal to surface osteoblasts and express histochemically detectable levels of alkaline phosphatase, one

of the earliest markers of the osteoblast phenotype. The preosteoblast starts to proliferate (*proliferation stage*) under the regulation of specific stimuli and a crucial signaling event occurs for the development of the large cuboidal osteoblasts on the bone surface. Osteoblasts are usually found in clusters of cuboidal cells along the bone surface, lining the layer of bone matrix that they are producing, before it is calcified. Activated mesenchymal cells and preosteoblasts can usually be found behind the osteoblast in one or two layers. Active osteoblasts have been shown to express receptors for parathyroid hormone, various cytokines and also exhibit different adhesion molecules (e.g. integrins) on their plasma membrane. Receptors for various steroid molecules such as estrogens and vitamin-D_3 also can be detected in osteoblasts. The active osteoblast is highly enriched in alkaline phosphatase (Figure 1), and secrets a large amount of type I collagen and specialized bone-matrix proteins as osteoid toward the mineralizing front of the tissue.

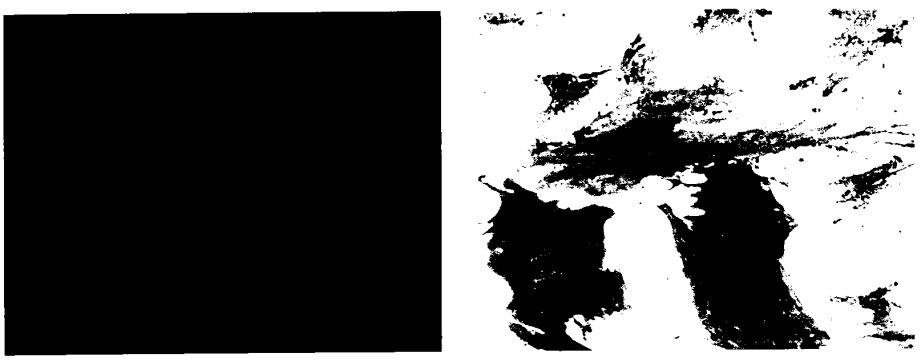

Figure 1.: Alkaline phosphatase activity of primary osteoblasts isolated from human bone marrow aspirates (Chapter 2, Materials and methods). Attached cells were stained *in situ* for AP with either Fast-red violet LB (left) or Fast blue (right) reagents, fixed with 2% buffered formalin (pH 7.4) and then analyzed by either fluorescent (left) or light (right) microscopy.

The collagen fibers must undergo a maturation process to support mineral deposition. The cytoskeleton transfers signals from the extracellular matrix through to the nucleus, which provides responsiveness of bone tissue to systemic factors and mechanical forces. These stimuli can influence the expression and regulation of bone-specific and bone-related genes in osteoblasts. The sequential expression of proteins involved in cell adhesion, extracellular

matrix biosynthesis and matrix mineralization provides a series of markers that indicate stages of osteoblast differentiation (Figure 2).

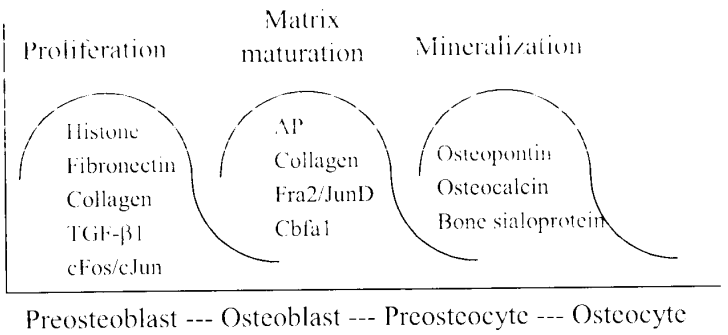

Figure 2.: Developmental phases of osteoblast differentiation. Spatial expression of osteoblast-related genes (structural molecules, growth factors and transcription factors) is shown in relation to the phenotype and functional stage[6]. (Figure is adopted with modifications from Stein G.S. et al.: Mechanisms regulating osteoblast proliferation and differentiation. in: Principles of bone biology, San Diego, Academic Press, 1996.)

The *proliferation stage* is inhibited by the accumulated extracellular matrix. As the cell growth decreases the *matrix-maturation stage* emerges, when the extracellular matrix is organized for mineral deposition by the induced expression of alkaline phosphatase and other bone-specific proteins. The *mineralization stage* leads to the expression of markers of the mature osteoblast (e.g. osteocalcin and bone sialoprotein), which facilitate the mineralization. This developmental sequence of gene expression can be shown in a single-cell level by in situ methods. Many of the osteoblast phenotypic markers (e.g. alkaline phosphatase and osteocalcin) can be examined in serum as useful markers of bone function. Toward the end of the mineralization period, the osteoblast becomes either a flat lining cell or an osteocyte. The osteoblast (osteocyte), however, may undergo apoptosis during this developmental process controlled by multiple osteotrop factors[5,6,10-12].

The organic extracellular bone matrix

Bone matrix is physiologically mineralized and is unique in that it is constantly regenerated throughout life as a consequence of bone turnover. Synthesis of the matrix components and the mineralization of the matrix are the primary functions of the osteoblast. The organic components of the bone matrix can be divided into two major groups: collagenous and non-collagenous proteins[11,13,14].

Collagens

The basic component of the bone matrix fiber system is type I collagen (90 % of the organic extracellular bone matrix), which is a triple helical molecule containing two identical α1[I] chains and a structurally similar, but genetically different α2[I] chain. Collagen α chains are characterized by a Gly-X-Y repeating triplet (where X is usually Proline), and by several posttranslational modifications including (1) hydroxylation of certain prolyl and lysyl residues, (2) glycosylation of certain lysyl or hydroxylysyl residues with glucose or galactose residues or both and (3) formation of intra- and intermolecular covalent cross-links. Measurement of these bone collagen cross-links in urine has proved to be good indicators of bone resorption. Bone consists predominantly of type I collagen, however, small amounts of type III, V, X collagens may be present during particular stages of bone formation[13,15].

Non-collagenous proteins

Approximately 10% of the organic bone matrix is composed by non-collagenous proteins. One fourth of this fraction is obtained exogenously from other tissues mainly composed of albumin and α_2-HS-glycoprotein. These proteins have impacts on matrix mineralization and α_2-HS-glycoprotein also may regulate bone cell proliferation. The remaining three fourth of this portion contains molecules produced locally by bone cells:

proteoglycans, glycosylated proteins, glycosylated proteins with potential cell-attachment activities and γ-carboxylated (gla) proteins[11,13].

1. Proteoglycans and hyaluronan

Proteoglycans are macromolecules that contain acidic polysaccharide side chains (glycosaminoglycans, e.g. *hyaluronan*) attached to a central core protein, and bone matrix contains several members of this family (*versican, decorin, biglycan, fibromodulin, osteoadherin*). Although, their exact role are yet to be fully explored, they are presumed to be important for the integrity of most connective tissue matrices by anchoring extracellular matrix molecules to each other and to bone cells, and also by facilitating mineralization[16,17].

2. Glycoproteins

The synthesis of high levels of *alkaline phosphatase* is one of the hallmarks of bone formation. This enzyme, primarily bound to the cell surface via a phosphoinozitol linkage, and can be cleaved from the cell surface and found within mineralized matrix. The exact functions of this enzyme are still not completely known. *Osteonectin* is a phosphorylated glycoprotein, which can be found in the greatest amount among the non-collagenous proteins of bone matrix. Its function in bone may be multiple, regulating osteoblast growth, proliferation, and also matrix mineralization. *Tetranectin* and *tenascin* also have been found in bone matrix, but their function is not yet known[14,18-20].

3. Glycosylated proteins with potential cell-attachment activities

Cells in all types of connective tissues interact with their extracellular milieu in response to stimuli that regulate specific cell functions, such as migration, proliferation and differentiation. Bone cells synthesize at least nine proteins that may have specific roles in cell attachment: *type I collagen, fibronectin, thrombospondins (I, II, III, IV), vitronectin, fibrillin, osteopontin, bone sialoprotein, BAG-75 and DMP-1*. All of these proteins contain RGD (Arg-Gly-Asn) triplets. This consensus amino acid sequence binds to the cell surface

expressed integrins. Both osteopontin and bone sialoprotein are known to anchor osteoclasts to bone and in addition to supporting cell attachment, bind Ca^{2+} with high affinity[13,14,19-21].

4. γ-carboxylated (gla) proteins

This group of non-collagenous proteins is characterized by the presence of dicarboxylic glutamyl (gla) residues. These proteins, *matrix-gla-protein*, *osteocalcin* (bone gla-protein) and *protein S* are posttranslationally modified by the action of vitamin-K-dependent γ-carboxylases. The production of dicarboxylic glutamyl residues enhances calcium binding. They probably function in the inhibition of mineral deposition. Osteocalcin deficient mice are reported to have increased bone mineral density. In human bone, it is concentrated in osteoblasts osteocytes and its release can be significantly increased by various osteotrop factors (e.g. vitamin-D). The secretion of these proteins may be a signal in the bone turnover cascade. Osteocalcin measurements in serum have proved valuable as a marker of bone turnover[14,20,22].

Bone remodeling

The adult skeleton is a highly specialized and dynamic organ that undergoes continuous regeneration by the coordinate actions of osteoclasts and osteoblasts on trabecular bone surfaces and in haversian system. Regeneration continues in the form of a periodic replacement of old bone with new at the same location. This process is called remodeling and is responsible for the complete regeneration of the adult skeleton in every 10 years. The most likely purpose of bone remodeling is to prevent accumulation of old bone.

The resorption of bone is the task of osteoclasts, and the formation of new bone is the job of osteoblasts. All osteoclasts and osteoblasts belong to a unique temporary structure, known as the basic multicellular unit (BMU). The BMU is approximately 1-2 mm long and 0.2-0.4 mm wide, comprises a team of osteoclasts in the front, a team of osteoblasts in the

rear, a central vascular capillary, a nerve supply and associated connective tissue. In human adults, 3-4 million BMUs are initiated per year, and about 1 million operating at any moment. The life span of the BMU is about 6 months, much longer than the life span of its cells. Therefore, continuous supply of osteoclast and osteoblasts from their progenitors is essential for an uninterrupted remodeling sequence. In the normal adult skeleton bone formation takes place only where bone resorption has previously occurred. The sequence of events at the remodeling site is the activation-resorption-formation order. Osteoclast activation is the initial step in the remodeling sequence, a process, which is not yet fully understood[12,23-25].

Recently, new members of the TNF family such as (1) the receptor activator of nuclear factor-kappaB (RANK), (2) RANK ligand (RANKL) and (3) the decoy receptor osteoprotegerin (OPG) for RANKL have been described and have been found to be essential regulators of bone remodeling, osteoclast differentiation and osteoclast activation. Osteoclasts express RANK, which interacts with either RANKL or soluble RANKL leading subsequently to osteoclast activation. RANKL is produced by a wide variety of cell types including osteoblasts, T cells and fibroblasts. The presence of OPG inhibits the interaction between RANKL and RANK by neutralizing RANKL and soluble RANKL, thus diminishes osteoclast development and function[26].

The osteoclast is the bone-lining cell responsible for bone resorption. The osteoclast is a giant multinucleated cell derives from haemopoetic cells in the monocyte/macrophage lineage. It is found in contact with a calcified bone surface within the Howship's lacuna that is the result of its own resorptive activity. This cell is rich in Golgi complexes, mitochondria, transport vesicles loaded with lysosomal enzymes. The cells are plentiful in ATPase, sodium-potassium pumps and exchangers of HCO_3^-/Cl^- and Na^+/H^+. The osteoclast synthesizes and excretes lysosomal enzymes and also metalloproteinases into the extracellular bone-resorbing compartment. Simultaneously, the osteoclast acidifies the extracellular compartment. The

extracellular bone-resorbing compartment is therefore the functional equivalent of a secondary lysosome with a low pH, enzymes and the substrate. First, the hydroxyapatite crystals are mobilized by digestion of their link to collagen (the non-collagenous proteins) and dissolved in the low pH. Then the residual collagen fibers are digested by either collagenases or cathepsins in the acidic microenvironment[27]. Therefore, the concentration of hydroxyproline and N-terminal collagen peptides in the urine can be used as indirect measurements of bone resorption (type I collagen is highly enriched in hydroxyproline and pyrinidoline links). The resorptive phase has been shown to last approximately 10 days. This period is followed by the repair of the defect by osteoblasts, which are attracted to site by chemotaxis then start to proliferate and make new bone (reversal, osteoid formation and mineralization phases). The duration of these phases has been experimentally measured, and it requires 3-6 months for the complete remodeling cycle (Figure 4).

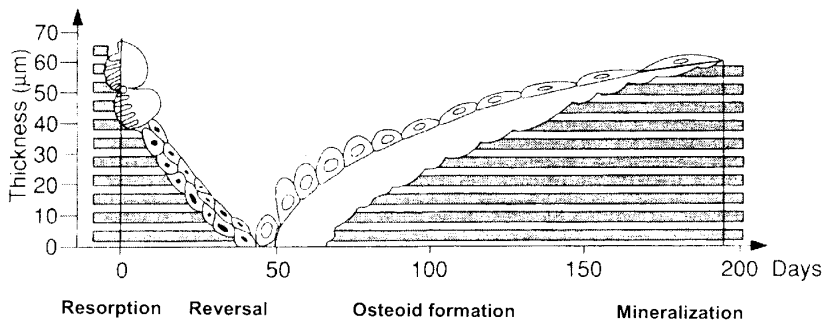

Figure 4.: Duration and depth of the different phases of the cancellous bone remodeling sequence. Axis *x* represents the duration and phases of the remodeling, while axis *y* demonstrates the mean wall thickness of a bone remodeling unit[24]. (Figure is adopted with modifications from Eriksen E.F. et al.: Bone histomorphometry, New York, Raven Press, 1994.)

Although cortical bone is anatomically different, its remodeling follows the same biological principles (Figure 5).

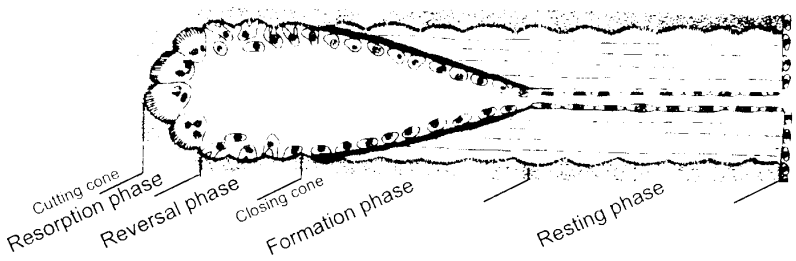

Figure 5.: The bone remodeling activity in cortical bone as a longitudinal sequence. Osteoclasts resorb the bone creating a cutting cone followed by the formation of new bone leading to the creation of a new bone structural unit (i.e. the haversian system)[28]. (Figure is adopted from Favus M.J.: Primer on the metabolic bone diseases and disorders of mineral metabolism, Philadelphia, Lippincott Williams and Wilkins, 1999.)

Cortical bone and cancellous bone, however, do not change with age in exactly the same way, thus they probably should be considered as two separate functional entities. Bone remodeling cells on cancellous bone surfaces are in direct contact with the cells of the bone marrow cavity, which produce a variety of osteotropic factors. Thus, it seems that osteoblasts and osteoclasts might be regulated primarily by these local compounds in cancellous bone. It is likely that cells in the cortical bone, which are more distant from the marrow cavity, are controlled more by the systemic osteotrop hormones[24,25,27,29].

The pathomechanism of periprosthetic osteolysis

Total joint replacement provides dramatic pain relief and improvement in joint function for patients with a variety of end-stage joint diseases. However, this mode of treatment is not without complications. The major long-term problems of total joint arthroplasty are periprosthetic osteolysis and aseptic implant loosening resulting in implant failure, bone stock deficiency, periprosthetic fractures and subsequent revision surgery[30-35] (Figure 5).

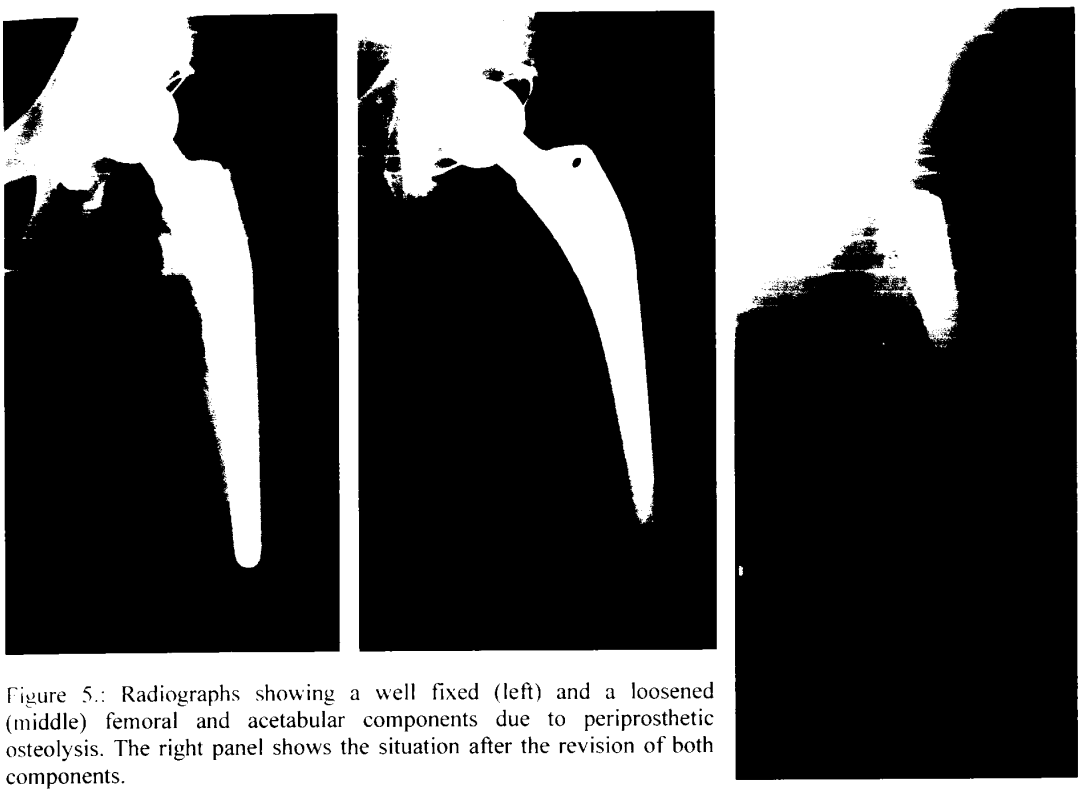

Figure 5.: Radiographs showing a well fixed (left) and a loosened (middle) femoral and acetabular components due to periprosthetic osteolysis. The right panel shows the situation after the revision of both components.

In the past decade, various theories have emerged concerning the pathogenesis of aseptic loosening and periprosthetic bone loss. These theories can be categorized into two different groups: mechanical theories and biological theories. The basic principle of the mechanical explanation is that micro- and macro-motions of the implant in concert with transiently elevated hydrodynamic pressure ultimately lead to periprosthetic bone necrosis and osteolysis (i.e. stress shielding)[32,36]. The biological models are based on the adverse cellular response to degradation products of implant materials due to corrosion or adhesive, abrasive and fatigue wear, which results in the generation of millions of particles (both polymeric and metal) annually[37-42] and in the release of metal ions into the periprosthetic space[43-45]. Multiple studies have investigated the properties of wear debris retrieved from failed cases. They found that the vast majority of particles are in the micron and sub-micron size range[38-42,46-48] with a wide variety of different shapes, and that approximately 20,000-40,000 particles are generated by each step (based on the volume loss of a polyethylene cup

of 55 mm^3/year)[37,41,49]. Furthermore, it has also been demonstrated that osteolytic areas contain ten times more particles that osteolysis-free areas from the same case ($3\text{-}4x10^6$ particles/g tissue vs. $30\text{-}40x10^6$ particles/g tissue)[37].

At revision surgery the presence of a granulomatous soft tissue membrane at the interface of bone and prosthesis (interfacial membrane, IFM) has been well-described[50-53]. This hyper-vascularized, aggressive, fibrotic tissue has unique properties resembling to rheumatoid synovial tissue, hence it is also called synovium-like membrane (SLM). Histological examination of this membrane has shown that there are distinct zones in IFM: (1) a synovial-like layer facing the cement/prosthesis surface, (2) the granulomatous stroma, which is a cell-rich area, and (3) a fibrous inner zone with collagen fibers and fibroblasts facing the bone bed. It has been revealed that the IFM is composed predominantly of macrophages, foreign body giant cells, histiocytes and fibroblasts, which contain or are in interaction with particles[39-42,54,55] (Figure 6, next page).

It is obvious that particle-cell interactions and particle phagocytosis is a prevalent phenomenon in the periprosthetic space *in-vivo*[56-59]. Phagocytosis is a host defense mechanism by which particles of different kinds can be eliminated[60-62]. However, the presence of non-degradable particles results in a constant state of activation of all types of cells in the periprosthetic space. Furthermore, high levels of different metals (in various ionic forms) have been detected in IFMs and in the serum of patients having loosened prosthetic component/s, contributing to further alterations of various cell functions[43,45,63,64]. These activated cells launch a cascade of "inflammatory processes" resulting in the formation of the above-described IFM.

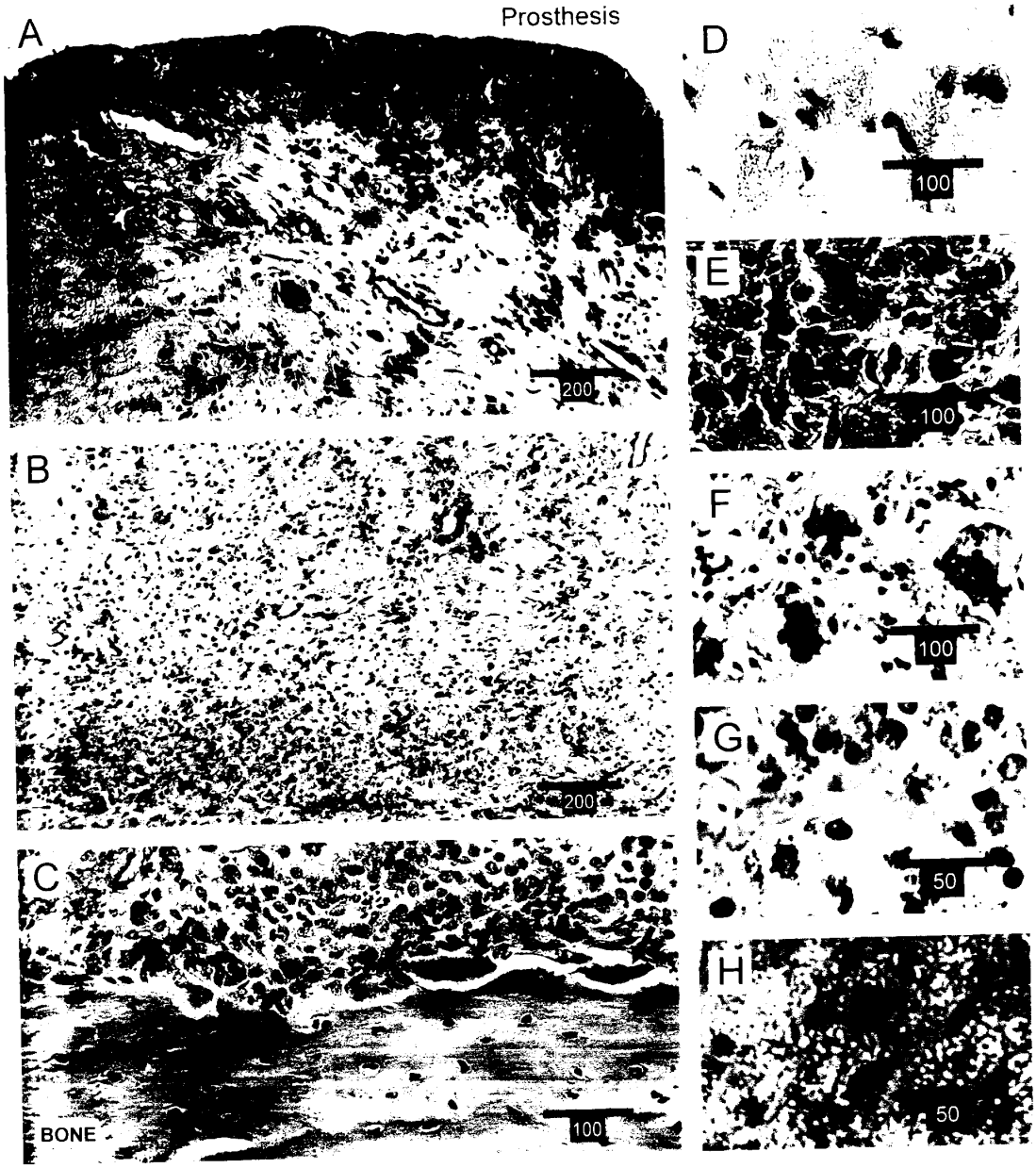

Figure 6.: Histology of IFM retrieved from failed total hip arthroplasty. This is a granulomatous, highly vascularized tissue with fibroblasts (B), osteoclasts (C, arrows), macrophages/histiocytes (E, G) foreign body giant cells (F). Fibrotic areas (A, B, D: blue) alternate with macrophage-rich areas that are adjacent to the prosthesis (A) or bone (C). Panel G shows macrophages with phagocytosed metal particles. Panel H demonstrates PE particles phagocytosed also by macrophages analyzed with polarized-light microscopy. Sections of panels A, B, D and E were stained with Weigert's trichrome staining in order to differentiate cells and extracellular matrix. Sections of C, F, G and H were stained with hematoxylin and eosin. Bars indicate size resolution in microns (μm).

Many studies have investigated the effects of particulate wear debris on the functions of monocytes/macrophages, fibroblasts and osteoclasts, all of which present in the periimplant space and are in contact with implant materials. It has been demonstrated that macrophages and fibroblasts produce proinflammatory cytokines (e.g. TNF-α, IL-1, IL-6), chemokines (MCP-1, IL-8), prostaglandins (PGE2) and matrix metalloproteases (MMPs; e.g. MMP-1, MMP-3)[55-57,65-74]. These factors are either activators of osteoclasts (e.g. TNF-α, IL-6)[75-80] or are directly responsible for bone matrix degradation (MMPs)[81-84]. More importantly, all of these mediators have been proved to present within the IFM retrieved from patients with failed implants confirming that similar events should take place *in-vivo*. Osteoclasts have been shown to be able to engulf particles, which results in their activation[59,85]. Taken together, the degradation of bone matrix by either osteoclasts or enzymes results in increased bone resorption in the periprosthetic milieu.

Hypothesis

While increased osteoblastic bone formation could replace the resorbed bone leading to a higher bone turnover but a normal density, this apparently does not happen *in-vivo* as the periprosthetic osteolytic process progresses. Therefore, we hypothesized that altered osteoblast function plays a crucial role in the development of periprosthetic bone loss in the presence of particulate wear debris, corrosion products and various cytokines. We tested this hypothesis by monitoring particle uptake of osteoblasts and by determining the effects of different particles, metal ions and cytokines/growth factors on several osteoblast functions. In Chapter 2, we analyzed the phagocytic activity, the expression of osteoblast-specific genes, production of bone matrix proteins, proliferation, viability and cytokine/growth factor release of human osteoblasts in the presence of various particulate wear debris and different cytokines/growth factors. Hence, we specified the basic behaviors of human osteoblasts in the

presence of implant degradation products. In Chapter 3, we identified basic intracellular signaling pathways responsible for those altered gene expression profiles and other distorted osteoblast functions.

In this study, it is suggested that the osteoblast may play a critical role in the pathogenesis of periprosthetic osteolysis by virtue of 1) osteoblasts in bone or osteoblast precursors in bone marrow are in close proximity or direct contact with prosthetic components or their degradation products; 2) altered osteoblast functions, induced by either prosthetic degradation products or soluble factors within the periprosthetic space, may contribute to bone loss by producing less matrix; and 3) stimulated osteoblasts may promote osteoclast differentiation and activation resulting in further bone loss.

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CHAPTER 2

The Effects of Particulate Wear Debris, Cytokines, and Growth Factors on the Functions of MG-63 Osteoblasts

Csaba Vermes, Raman Chandrasekaran, Joshua J. Jacobs, Jorge O. Galante, Kenneth A. Roebuck and Tibor T. Glant

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Abstract

Background: Particle-challenged cells release cytokines, chemokines and eicosanoids, which contribute to periprosthetic osteolysis. The particle-induced activation of macrophages and monocytes has been extensively studied, but only limited information is available on the response of osteoblasts to particulate wear debris. This study examines the effects of particulate wear debris and/or proinflammatory cytokines and growth factors on osteoblast functions.

Methods: MG-63 osteoblasts were treated with metal (titanium, titanium-alloy and chromium orthophosphate) or polymeric particles (polyethylene and polystyrene) of phagocytosable sizes, or with exogenous cytokines and growth factors. The kinetics of particle phagocytosis and the number of engulfed particles were assessed using fluorescinated particles. Cell proliferation was determined by ^{3}H-thymidine incorporation and cell viability by either fluorescein diacetate uptake or trypane blue exclusion. Expressions of osteoblast-specific genes were quantified by Northern blot hybridization, and the secretions of osteoblast-specific proteins and cytokines were analyzed by enzyme-linked immunosorbent assays.

Results: MG-63 osteoblasts phagocytosed particles and become saturated after 24 hours. A maximum of 50-60 particles per cell were phagocytosed. Each type of particle

significantly ($p<0.05$) suppressed procollagen $\alpha 1$[I] gene expression, whereas other osteoblast-specific genes (osteonectin, osteocalcin and alkaline phosphatase) did not show significant changes. Particle-stimulated osteoblasts released interleukin-6 (IL-6) ($p<0.05$) and a smaller amount of transforming growth factor-$\beta 1$ (TGF-$\beta 1$). Particles reduced ($p<0.05$) cell proliferation in a dose dependent manner without affecting cell viability. Exogenous tumor necrosis factor-α (TNF-α) also enhanced IL-6 ($p<0.01$) and TGF-$\beta 1$ ($p<0.05$) release, whereas TGF-$\beta 1$ secretion was increased by IGF-I and prostaglandin-E2 as well. IGF-I and TGF-$\beta 1$ significantly ($p<0.05$) increased, while TNF-α and prostaglandin-E2 significantly ($p<0.01$) suppressed procollagen $\alpha 1$[I] gene expression in osteoblasts. In contrast, neither exogenous nor endogenous IL-6 had any effect on other cytokine secretion, proliferation or on procollagen $\alpha 1$[I] gene expression in MG-63 osteoblasts. Transcription inhibitor actinomycin D reduced both procollagen $\alpha 1$[I] transcription and IL-6 production. Inhibitors of protein synthesis (cyclohexamide) and intracellular protein transport (brefeldin A, monensin) blocked IL-6 release, but none of these compounds influenced the suppressive effect of titanium on procollagen $\alpha 1$[I] gene expression.

Conclusions: MG-63 osteoblasts phagocytose particulate wear debris, and this process induces IL-6 production and suppresses type I collagen synthesis. Osteoblast-derived IL-6 may induce osteoclast differentiation and/or activation, but the resorbed bone cannot be replaced by new bone due to diminished osteoblast function (reduced type I collagen synthesis). Exogenous cytokines (TNF-α and IL-1β), growth factors (IGF-I and TGF-$\beta 1$) and prostaglandin-E2 can modify particulate-induced altered osteoblast functions.

Clinical relevance: Altered osteoblast functions probably contribute to the progression of periprosthetic osteolysis. Suppressed osteoblast functions, however, could be compensated by certain growth factors such as IGF-I or TGF $\beta 1$. These growth factors, if

delivered locally, may have therapeutic potential to prevent or reverse periprosthetic osteolysis.

Introduction

Periprosthetic osteolysis is a major clinical problem, which may jeopardize the long term success of total joint arthroplasty[1-4]. Typically, a granulomatous tissue of fibroblasts, macrophages and foreign body giant cells develops at the interface of bone and prostheses or bone and bone cement[5-8]. All cell types of this interfacial tissue contain wear debris from prosthetic components[9-14], and particle phagocytosis is a central event in the pathogenesis of periprosthetic osteolysis[15-18].

Phagocytosis is a non-specific defense mechanism for the elimination of tissue debris, bacteria and foreign particles. The phagocytic process requires the opsonization of particles and the protein coat (specific or non-specific proteins, immunoglobulins, complement) on the surface of particles binds to "phagocytosis receptors" (e.g. Fcγ receptors, complement or mannose receptors, β1 integrins) and this interaction activates intracellular signaling pathways that lead to cytoskeletal reorganization, pseudopod formation and the ingestion of particles[19-21]. This phagocytosis-induced signaling process may simultaneously result in up- or downregulation of a number of genes via the action of various nuclear transcription factors[20,22-25]. However, the normal phagocytic process may be altered when tissue macrophages, or other cells, are continuously exposed to non-degradable wear debris.

The phagocytosis of particulate wear debris stimulates macrophages/monocytes to secrete mediators of bone resorption such as eicosanoids, interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) *in vitro*[12,15,22,25-29]. These compounds have also been shown to be present in the periprosthetic soft tissue *in vivo*[8,12,30].

Osteoblasts, a key cell type involved in bone remodeling, also phagocytose particles. This process upregulates the release of cytokines[23] and prostaglandin-E2[31] inducing bone loss

via osteoclast activation. In addition, particle-stimulated osteoblasts exhibit suppressed procollagen α1[I] gene expression followed by reduced type I collagen synthesis[17,23], which may result in decreased bone formation. Thus, particle-induced altered osteoblast functions may play a critical role in pathological bone resorption via both osteoclast activation and reduced osteoblastic bone formation. In previous studies, we have identified the upstream signaling events in particle-stimulated osteoblasts[23], which ultimately led to the suppression of procollagen α1[I] gene[17,23]. Activation of protein tyrosine kinases seems to be the earliest cellular event resulting in the activation of nuclear transcription factor-kappaB (NF-κB)[23] in osteoblasts, and this transcription factor was shown to be activated in particle-challenged human macrophages as well[22]. NF-κB, however, is a general transcription factor, which may suppress procollagen α1[I] mRNA[23,32], while simultaneously upregulating many other genes including the genes of proinflammatory cytokines such as IL-1, IL-6 and TNF-α[22] in particle-challenged cells. Since these mediators are continuously secreted by particle-challenged cells, their effects are probably crucial in the development of periprosthetic osteolysis by altering osteoblast and osteoclast functions.

We hypothesize that besides the direct effects of particulate wear debris on osteoblasts (increased IL-6 release and suppressed type I collagen synthesis)[23] there is a paracrine regulation of cytokines, prostaglandins and growth factors, which contribute to bone resorption by alteration of osteoblast functions. We investigated this hypothesis by monitoring the kinetics of particle phagocytosis in osteoblasts and determining the effects of proinflammatory cytokines and growth factors on osteoblast specific gene expressions, cell proliferation and cytokine release.

Materials and Methods

Particles

All metal and polymeric particles used in these studies were described previously[10,17,26,29]. Particles of commercially pure titanium with 1.3 micrometer nominal diameter and chromium-orthophosphate ($CrPO_4x4H_2O$; 1.42 ± 0.83 micrometers)[29] were purchased from Johnson Matthey (Danvers, MA). Titanium-alloy (6 percent aluminum-4 percent vanadium) particles (Sulzer Plasma Technik, Troy, MI) were ground from a 150-300 micrometer size grinding particles[27]. Conventional medical grade ultra high molecular weight polyethylene (GUR 415, Hoecht-Celanese, Houston, TX) was pulverized by in liquid nitrogen[27]. Particles were sedimented and filtrated for size separation. Particles exhibited a comparable size distribution. At least 90 percent were smaller than three micrometers in diameter. Polystyrene (1.14 ± 0.01 micrometers) and polystyrene based fluorescent Fluoresbrites (0.926 ± 0.027 micrometers) particles were purchased from Polyscience (Warrington, PA). Based upon the size distribution, a 0.1 percent (volume/volume) particle suspension contained approximately $2.2\text{-}6.7x10^8$ particles/milliliter. Particles were sterilized by irradiation with 2.2 megarad (22,000 gray) from a Cs-137 source (model 143; J.L. Sheppherd Irradiator, San Fernando, CA), opsonized in 10 percent human type AB serum[27] and stored in sterile phosphate-buffered saline (pH 7.2). Endotoxin contamination of particles was excluded by limulus assay (E-Toxate, Sigma Chemical, St. Louis, MO).

Cells and cell cultures

The MG-63 osteoblast cell line was purchased from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in monolayer in Dulbecco's modified Eagle medium (GIBCO, Grand Island, N.Y.) containing 10 percent fetal bovine serum

(Hyclone Laboratories, Inc., Logan, UT) in humidified athmosphere of 5 percent carbon dioxide in air at 37 degrees centigrade[16,17,26].

Treatment of cells with particles, cytokines and growth factors

Confluent cultures of cells were subjected to serum starvation (0.3 percent fetal bovine serum) for 24 hours prior to treatment. Culture media were then replaced with fresh medium containing 0.3 percent fetal bovine serum and particles, cytokines or growth factors. Proliferation and viability assays, phagocytosis analysis and total RNA extraction were carried out on the cultured cells. Tissue culture media were collected at various time points, centrifuged, filtered through a 0.22 micrometer polycarbonate filter (Spin-x; Costar, Cambridge, MA) and stored at -80 degrees centigrade. All experiments were performed in duplicate or triplicate in at least five independent experiments.

Reagents were purchased from Calbiochem (La Jolla, CA) or R&D Systems (Minneapolis, MN). Insulin-like growth factor-I (IGF-I; 30 nanogram per milliliter) and transforming growth factor-beta1 (TGF-β1, 20 picogram per milliliter) were used to stimulate collagen production[33,34]. TNF-α, IL-6 and IL-1β were added to the cultures at concentrations of 10 nanogram per milliliter, 500 picogram per milliliter and 30 picogram per milliliter. Prostaglandin-E2 (100 nanogram per milliliter; prostaglandin-E2) is an eicosanoid, which was shown to regulate collagen synthesis[35]. Actinomycin D (1 microgram per milliliter) was used to block transcriptional events, cyclohexamide (35.5 micromolar) to inhibit protein translation and synthesis, brefeldin A (0.1 micromolar) to inhibit the transport of freshly synthesized proteins from the endoplasmatic reticulum to Golgi apparatus, monensin (1.5 micromolar) to block the release of newly synthesized proteins from Golgi, and Cytochalasin D (1 micromolar) to destabilize the cytoskeleton, thus inhibiting phagocytosis. All of the

above listed concentrations were selected after serial dilutions of each compound tested in MG-63 cell culture.

Viability tests and ^{3}H-thymidine incorporation

The trypan blue exclusion test was used to determine the viability of cells. Since the presence of phagocytosed titanium particles, especially at higher concentrations, precluded a precise evaluation of dye-exclusion, cell viability was also determined with fluorescein diacetate (Molecular Probes, Eugene, OR)[29]. Viability tests were performed in duplicate and at least 200 cells were counted using transmission light (for trypan blue exclusion) or fluorescent microscopy (for fluorescein diacetate) in a Nikon Microphot-FXA microscope (Nikon, Tokyo, Japan).

Proliferation of cells was measured by ^{3}H-thymidine (Amersham International, Arlington Heights, IL) incorporation into DNA in a 96-well microplate system. Trypsinized cells were harvested (Tomtec Inc., Orange, CT) at different time points after a 12 hour ^{3}H-thyimidine (1 micro-Curie of ^{3}H-thymidine per well) incubation.

RNA extraction and Northern blot hybridization

Total RNA samples were isolated from monolayer cultures as described[16,17]. Approximately 10 micrograms of total RNA was denatured in 50 percent formamide and 17.5 percent formaldehyde, dissolved in MOPS buffer (20 millimolar 3-[N-morpholino]propane-sulfonic acid, 5 millimolar sodium acetate and 1 millimolar ethylenediaminetetraacetic acid at pH 7.0), separated by electrophoresis in 1 percent agarose gel and then transferred to GeneScreen Plus membranes (New England Nuclear, Boston, MA). Blots were hybridized with ^{32}P-dCTP-labeled specific cDNA probes at a concentration of $3x10^{6}$ counts per minute per milliliter (specific activities: $2\text{-}6x10^{8}$ counts per minute per microgram cDNA)[16]. Human-

specific cDNA probes (plasmids) were purchased from ATCC. The following recombinant plasmid DNAs were used as probes: a 1.8 kilobase long cDNA probe for procollagen α1[I] (Hf677; ATCC #61322), a 2.0 kilobase probe for osteocalcin (ATCC #86269), a 1.8 kilobase probe for osteonectin (ATCC #78193) and a 1.5 kilobase probe for alkaline phosphatase (ATCC #59633). Following hybridization, the blots were washed and exposed to Kodak XAR-5 film (Kodak, Rochester, NY) at -70 degrees centigrade for photographic documentation or the original Northern blot was analyzed by STORM PhosphorImiger using ImigeQuant software (Molecular Dynamics, Sunnyvale, CA).

Measurement of particle phagocytosis

To further understand particle-induced changes in osteoblast function, we characterized the kinetics of particle uptake by MG-63 cells. In initial experiments, cells grown in monolayers were treated with 0.1 percent (volume/volume) titanium particles for various time periods, harvested by trypsinization, washed, and allowed to attached to coverslips. Trypsinization and repeated washing of cells removed non-phagocytosed and/or surface attached particles, and cell-free areas of the coverslip had essentially no particles. Two hundred particle-treated cells from different areas on the coverslip were examined by light microscopy (Nikon). Although we could easily identify cells containing particles, it was difficult to determine the exact particle number in a single cell due to intracellular aggregation of titanium particles.

To circumvent this problem and to be able to quantify the number of phagocytosed particles within a single cell, we used fluorescent particles (Fluoresbrite) as an alternative particle source. Two different methods were applied to determine the number of engulfed particles in osteoblasts. First, the number of Fluoresbrite particles in cells was counted directly in epifluorescence mode using Nikon Microphot-FXA microscope. However, this

method could only be applied to cells treated for short time periods (less than 12 hours), as the large number of phagocytosed particles in cells treated for longer periods of time formed intracellular aggregates precluding accurate particle counts. The second method involved preparing serial dilutions of Fluoresbrite particles. Particle numbers were counted in a hemocytometer and the fluorescence intensity of each concentration determined by a fluorescent plate reader (Victor 1420 Multilabel Counter, Wallac, Gaithersburg, MD). Particle numbers and the corresponding fluorescence intensities were plotted to generate a standard curve, which then was used to determine the number of fluorescent particles (based on the fluorescence intensities) in cell lysates from osteoblasts treated with Fluoresbrite. Cells containing phagocytosed Fluoresbrite were trypsinized, washed, counted and lysed by ultrasonication (Virtis, Gardina, NY) at 20 kilohertz for 2 minutes on ice.

Measurement of cytokines and osteoblast-specific proteins in culture media

Cytokine concentrations in supernatants of osteoblast cultures were measured by sandwich enzyme-linked immunosorbent assays (ELISA) in 96-well microtitration plates following the manufacturer's instructions. High sensitivity assay kits for TNF-α (assay range from 0.5 to 32 picogram per milliliter), IL-1β, IL-6 (sensitivity range for both from 0.12 to 8.0 picogram per milliliter) and TGF-β1 (range from 31 to 2000 picogram per milliliter) were purchased from R&D Systems. Secreted osteocalcin was measured by NovoCalcin and type I collagen by Procollagen-C ELISAs purchased from Metra Biosystems (Mountain View, CA).

Statistical analysis

Descriptive statistics were used to determine group means and standard deviations. The Pillai's trace (similar to Wilks' lambda or Hotelling-Lawley's trace) criterion was used to

detect multivariate significance. Subsequently, paired Student's t tests were performed between groups of interest. The level of significance was set at $p<0.05$. All statistical analyses were performed using computer-based statistical software (SPSS/PC+ v 4.0.1, SPSS Inc, Chicago, IL).

Results

Particle phagocytosis by osteoblasts

Osteoblasts phagocytosed particles in a time-dependent fashion (Fig. 1). To determine the number of phagocytosed particles, however, is a difficult issue due to the indiscernible location of phagocytosed, partially engulfed or surface-"attached" particles, and the intracellular aggregation of phagocytosed particles. The use of fluorescent particles (Fluoresbrite) is a method for determining the number of engulfed particles. The average particle number per cell, measured by fluorescent intensity in MG-63 cell lysates, ranged from 0 to 2 (mean 0.4 ± 0.6 S.D.) after one hour, 3-12 (5 ± 2) after 2 hours, 7-19 (13 ± 3) after 6 hours, 16-28 (23 ± 2) after 12 hours, 53 ± 5 after 24 hours, 60 ± 6 after 48 hours and 58 ± 5 after 72 hours (Fig. 1-C). A maximum of 40-60 Fluoresbrite particles phagocytosed within 24 hours seems to be the saturation level for MG-63 cells. The number of engulfed particles could be precisely determined only when fluorescent-labeled particles were used. Cytochalasin D, which destabilizes the cytoskeleton and inhibits phagocytosis, significantly reduced (2-10, 4 ± 3; after 48 hours), but did not completely abolish the phagocytosis of particles (Figs. 1-A2 and 1-B2).

Effect of particulate wear debris on cell viability and proliferation

We next addressed how the phagocytosed particles influenced cell functions and whether differing composition of particles can initiate distinct cell responses. Particulate wear

debris had no effect on cell viability of MG-63 cells, which remained higher than 95 percent even in long-term (72-96 hours) experiments over a wide range of particle composition (titanium, titanium alloy, chromium orthophosphate, polyethylene, polystyrene or Fluoresbrite) and concentrations (0.0125-0.2 percent volume/volume). In contrast to viability, cell proliferation was reduced compared to untreated cultures, and the effect was dose dependent (Fig. 2-A). Interestingly, suppressed proliferation became normalized at low particle concentrations (0.0125 percent to 0.05 percent) by 48 hours (Fig. 2-A).

Effects of exogenous cytokines on cell viability and proliferation

Exogenous cytokines (IL-1β and IL-6), prostaglandin-E2 and growth factors TGF-β1 and IGF-I had no effect on cell viability over a wide range of concentrations (data not shown). Only TNF-α reduced significantly cell viability ($p<0.01$) when higher concentrations (over 100 nanogram per milliliter) were used, especially in long-term (grater than 72 hours) experiments. As shown in Fig. 2-B, TNF-α (at a non-toxic concentration of 10 nanogram per milliliter) and prostaglandin-E2 decreased, while TGF-β1 and IGF-I increased cell proliferation. Neither IL-1β nor IL-6 affected cell proliferation.

Cytokine release in osteoblasts induced by either titanium particles or exogenous cytokines

The earliest cytokine release in culture media of titanium-stimulated MG-63 cells was detected after 12 hours and it was restricted to IL-6 (Fig. 3-A). There were undetectable amounts of IL-1 and TNF-α in culture media of either untreated or particle-challenged MG-63 cells. A basal TGF-β1 secretion was enhanced in particle-treated cultures after 72 hours (Fig. 3-B).

As found in titanium-stimulated MG-63 cultures, only IL-6 and TGF-β1 secretion were modified by exogenous mediators. Only TNF-α at a non-toxic (10 nanogram per

milliliter) concentration had a significant ($p<0.01$) effect on IL-6 release (Fig. 4-A), whereas the basal TGF-β1 secretion was increased ($p<0.05$) by exogenous TNF-α, IGF-I or prostaglandin-E2 (Fig. 4-B).

Suppression of osteoblast-specific genes by particulate wear debris

We have reported a 40-60 percent suppression of procollagen α1[I] mRNA expression in MG-63 osteoblasts exposed to titanium particles[17,23]. To further characterize the effect of particles on osteoblast-specific gene expression and protein synthesis, MG-63 cells were exposed to titanium, titanium alloy, chromium orthophosphate, polystyrene, polyethylene and Fluoresbrite particles for 48 hours. Particles, regardless of composition, significantly ($p<0.05$) suppressed procollagen α1[I] mRNA expression in MG-63 osteoblasts (Fig. 5). This downregulation of collagen gene expression was accompanied by reduced type I collagen protein synthesis. In contrast to procollagen gene expression, none of the particles significantly altered the expression of osteocalcin or other osteoblast-specific genes such as osteonectin or alkaline phosphatase (Fig. 5). These data demonstrate that particles differentially affect gene expression in osteoblasts (for example procollagen α1[I] versus osteocalcin) and the gene-specific effect is a general response to particles and not specific to particles of a particular composition.

Effect of exogenous cytokines upon procollagen α1[I] gene expression

An increased IL-6 secretion in titanium-stimulated osteoblasts (Fig. 3-A) correlated inversely with the suppression of procollagen α1[I] mRNA and reduced collagen synthesis in all particulate-stimulated osteoblast cultures. Neither exogenous IL-6 (Fig. 6-A) nor neutralizing antibodies to IL-6 (data not shown) altered the collagen gene expression either in the presence or absence of titanium particles indicating that procollagen α1[I] gene regulation

was independent of IL-6 or IL-6 induced transcription factors in osteoblasts. In contrast, the effect of exogenous TNF-α on procollagen α1[I] gene expression was highly comparable to the effect of titanium particles (Fig. 6-A) and this correlated with reduced type I collagen synthesis[23]. Prostaglandin-E2 also inhibited procollagen α1[I] gene expression (not shown). Exogenous IL-1β significantly ($p<0.05$) reversed titanium induced procollagen α1[I] gene suppression (Fig. 6-A). Growth factors IGF-I and TGF-β1 significantly ($p<0.05$) increased the collagen gene expression and could completely reverse the titanium-induced suppression of procollagen α1[I] mRNA (Fig. 6-B).

The effect of transcriptional, translational and protein transport inhibitors on cytokine release and procollagen α1[I] gene expression

In order to determine whether an effect of a freshly synthesized cytokine was involved in the titanium-induced procollagen α1[I] gene suppression and to distinguish the mechanism of titanium-induced IL-6 production from procollagen α1[I] gene suppression at the molecular level, MG-63 cells were treated with various inhibitors prior to stimulation with titanium particles. Actinomycin D, a potent inhibitor of transcriptional events by inhibiting RNA polymerase II, was used as a positive control. This compound blocked both the procollagen α1[I] mRNA transcription and IL-6 production. Translational (protein synthesis) inhibitor cyclohexamide, protein transport inhibitor brefeldin A, and monensin, a non-selective inhibitor of the release of newly synthesized protein from the Golgi apparatus, uniformly blocked the IL-6 release. In contrast, none of these chemicals modified titanium particle-induced suppression of procollagen α1[I] mRNA level (Fig. 7), confirming our recent observation that particle phagocytosis has a direct effect on procollagen gene expression via the activation of protein tyrosine kinase-NF-κB pathway[23]. Taken together, particle phagocytosis has a direct effect at the transcriptional level upon procollagen α1[I]

mRNA, whereas particle-induced cytokine release requires factors for protein synthesis and intracellular trafficking.

Discussion

Particulate wear debris from prosthetic components are continuously generated and phagocytosed by cells of the periprosthetic soft tissue. Phagocytosis is a strong signal for cells, first inducing a series of upstream events of cell stimulation via the activation of protein tyrosine kinases[20,23,24,36,37]. Activation of protein tyrosine kinases leads to the activation of nuclear transcription factors, which then are translocated into the nucleus with the result of upregulation of various genes, including proinflammatory cytokines. In a broader sense, all cell types of the periprosthetic soft tissue (macrophages, fibroblasts, osteoclasts and osteoblasts) are able to phagocytose particulate wear debris and virtually all cells can reach an activated state. These cells produce a number of cytokines, chemokines and prostaglandins, which may further affect the function of cells in either an autocrine or paracrine manner using distinct signaling mechanisms.

Bone is a dynamic tissue with a well-balanced homeostasis preserved by coupled bone formation and bone resorption. Normal bone turnover, however, can be unbalanced by either increased osteoclast activity or diminished osteoblast function; either or both mechanisms may result in net bone loss. Both cell types (osteoclasts and osteoblasts) phagocytose particles *in vitro*, and it is assumed that this may occur *in vivo* as well. Osteoblasts, while phagocytose particles become activated and produce IL-6[23] and prostaglandin-E2[31], simultaneously loosing their capacity to synthesize type I collagen[17,23]. The secreted IL-6[38,12] and prostaglandin E2[42,43] then activate osteoclasts in a paracrine fashion, which cells are assumed to be already in an activated state due to phagocytosed particles at the interface[18,44]. Other cytokines such as IL-1β and TNF-α, secreted by particle-stimulated macrophages/monocytes[8,12,15,22,26-30], are also present in the periprosthetic tissue.

TNF-α can activate osteoblasts to secrete IL-6 and suppress type I collagen synthesis[23,45,46], similar to the effect described for particle phagocytosis (Figs. 3-A, 4-A and 6-A). In addition, both IL-1β and TNF-α induce osteoclast differentiation from precursors and activate differentiated osteoclasts *in vitro*[40,42,43]. Taken together, phagocytosis directly, and phagocytosis-induced cytokine release indirectly, affect the bone turnover negatively via alteration of osteoblast and osteoclast functions.

From the osteoblast side, a phagocytosis-induced direct signal and exogenous TNF-α (paracrine effect) seem to be the most potent inducers of diminished type I collagen synthesis. The particle- and TNF-α-induced signaling mechanisms, however, must be independent because (1) we were unable to detect TNF-α in either particle-treated MG-63 (Fig. 3) or bone marrow derived primary human osteoblast cultures[23], (2) neutralizing anti-TNF-α antibody could abolish the exogenous TNF-α effect but did not modify the osteoblast response to titanium particles (not shown), and (3) protein synthesis inhibitors, while blocking cytokine release, had no influence on particle-induced procollagen α1[I] gene suppression (Fig. 7).

While neither exogenous nor endogenous IL-6 can affect osteoblast-specific functions, IL-6 along with IL-6 soluble receptor could enhance different osteoblast responses[47-49]. The IL-6 receptor complex consists of two transmembrane proteins: a ligand-binding chain (IL-6 receptor) and a non-ligand binding signal transducer, glicoprotein 130 (gp130). IL-6 binding to the ligand-binding chain triggers the heterodimerization of the two chains, and then the cytoplasmic domain of the gp130 chain transduces the signal. The soluble form of the ligand binding chain (soluble IL-6 receptor) is also able to activate gp130 when IL-6 binds to it. It is likely that MG-63 osteoblasts express gp130, but not the ligand binding chain[47,48], therefore neither secreted nor exogenous IL-6 can bind to IL-6 receptor, hence no signal can be transferred from the cell surface to the cell[41,47-50].

Among a number of cytokines and growth factors, IGF-I and TGF-β1 were able to completely reverse the suppressive effect of particles on procollagen α1[I] gene expression. These growth factors, when used alone, significantly upregulated the procollagen α1[I] gene expression (Fig. 6-B) and type I collagen synthesis. Furthermore, these growth factors increased osteoblast proliferation without affecting cell viability and inducing substantial IL-6 secretion. Thus IGF-I and TGF-β1 seem to be potent inducers of bone matrix formation.

One of the most important findings of this study is that, in addition to a direct effect of particles on osteoblast functions[17,23], the proinflammatory cytokine TNF-α also exhibits a massive and substantial effect on procollagen α1[I] gene expression, cell proliferation, cell viability and IL-6 secretion in osteoblasts. While this proinflammatory cytokine induces bone resorption via osteoclast activation, it also contributes to bone loss via reduced bone formation by osteoblasts. Since TNF-α, IL-1β and IL-6 are present and continuously secreted by particle-stimulated cells in the periprosthetic space, their long-term *in vivo* autocrine and paracrine effects are critical in the pathogenesis of the periprosthetic osteolysis. Eventually, local delivery of certain growth factors (IGF-I or TGF-β1), protein tyrosine kinase or NF-κB inhibitors[23], all of which can reverse the suppressive effect of either proinflammatory cytokines or wear particles on type I collagen synthesis in osteoblasts, may have a therapeutic potential to prevent or treat periprosthetic bone loss.

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Figures and figure legends

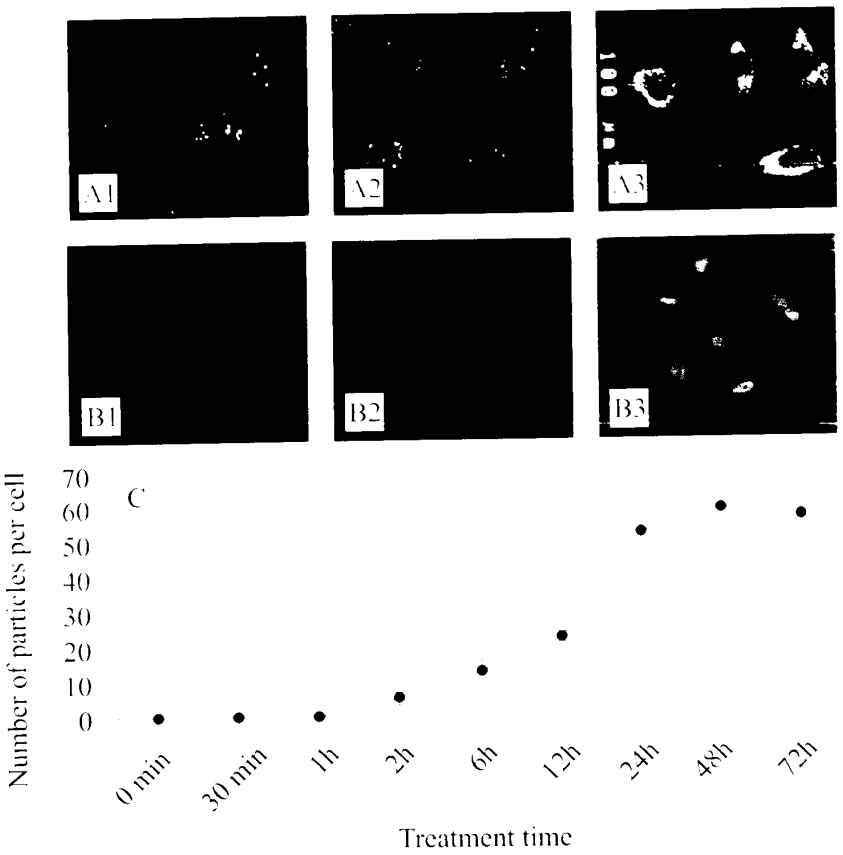

Figure 1.: Phagocytosis of particles by MG-63 osteoblast cells. Cells were cultured in Petri-dishes, serum deprived and then exposed to either Fluoresbrite (A) or titanium (B) particles for 2 hours (A1 and B1), or for 48 hours in the presence (A2 and B2) or absence (A3 and B3) of Cytochalasin D. Cells were harvested, washed, seeded on coverslips and fixed in 10 percent formalin. Fixed cells were counterstained with crystal-violet and examined by fluorescence microscopy (panels A and B). The number of phagocytosed Fluoresbrite particles in a single cell is shown in panel C at different time points.

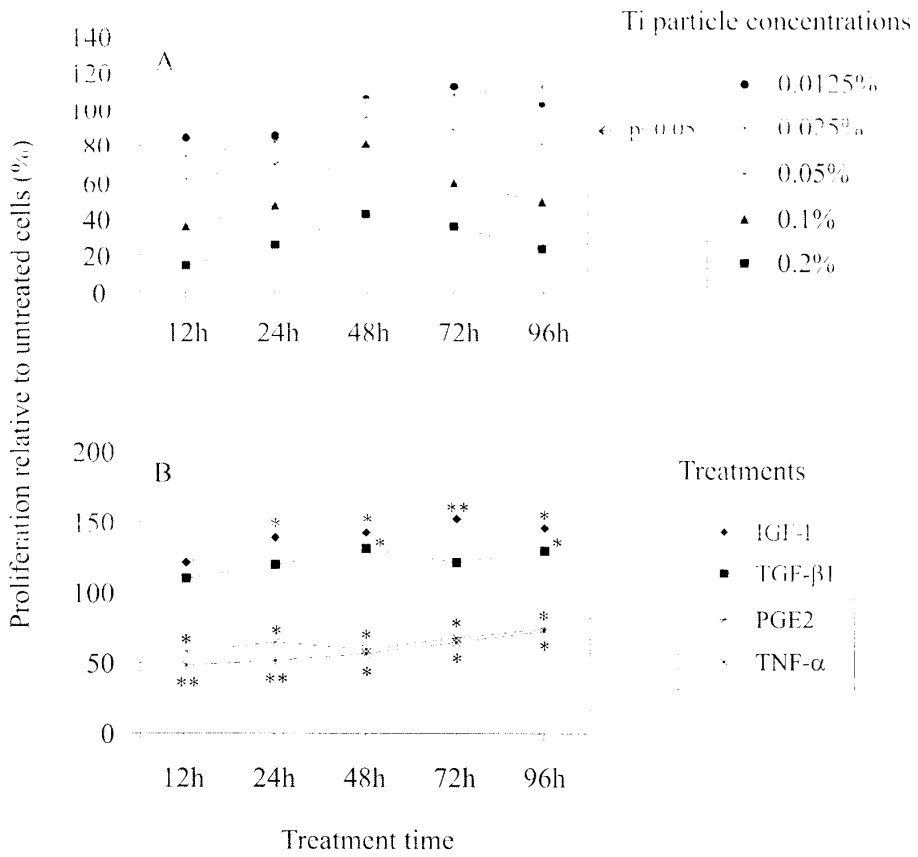

Figure 2.: The effect of titanium particles and exogenous mediators on MG-63 cell proliferation. Cells were untreated or treated either with different concentrations of titanium particles (panel A) or exogenous cytokines and growth factors (panel B). The level of proliferation was measured by ^{3}H-thymidine incorporation and was normalized to untreated samples (100 percent) at each time point. Error bars are omitted for clarity, but broken line indicates significance at a minimum of $p<0.05$ level on panel A. Levels of significance (relative to untreated cells) on panel B are shown: * $p<0.05$ and ** $p<0.01$.

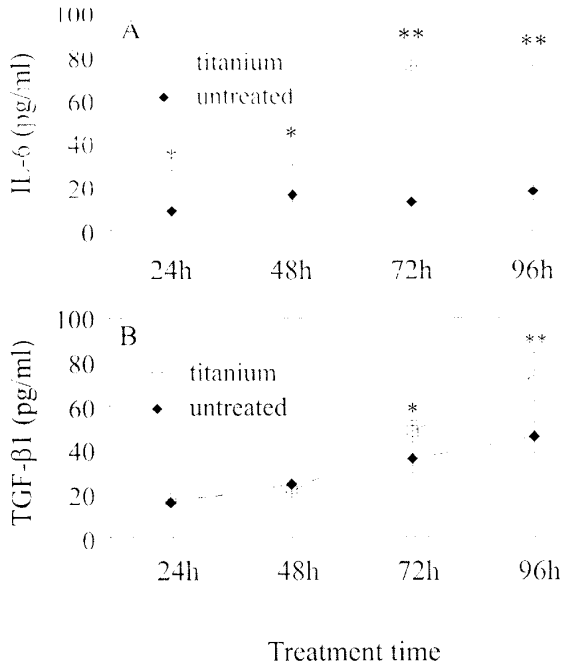

Figure 3.: Effect of titanium particles on IL-6 and TGF-β1 production by MG-63 cells. Confluent osteoblast cultures were either untreated or treated with 0.1 percent (volume/volume) titanium particles, and levels of IL-6, TNF-α, IL-1β and TGF-β1 were measured by ELISA at various time points. Note that only IL-6 (panel A) and TGF-β1 (panel B) reached detectable and significantly elevated levels as indicated: * $p<0.05$ and ** $p<0.01$.

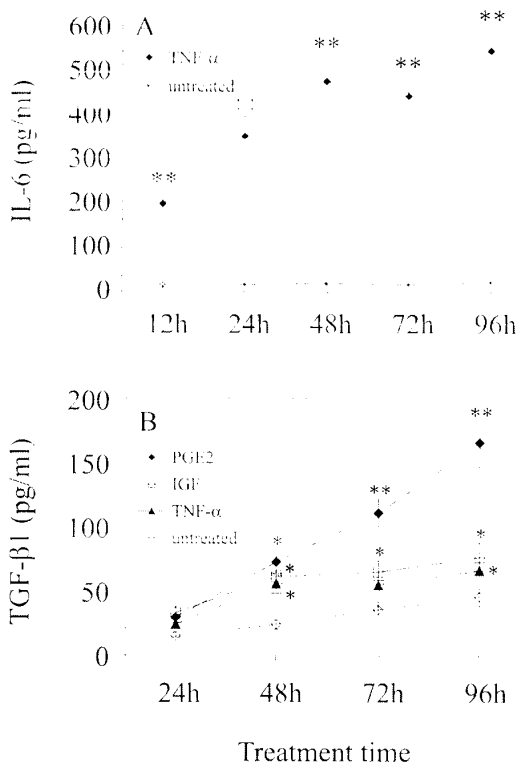

Figure 4.: Effect of exogenous cytokines on the release of cytokines by MG-63 cells. Confluent osteoblast cultures were untreated or treated with exogenous IL-6, TNF-α, IL-1β, TGF-β1, IGF-I or prostaglandin-E2 as described in Methods. Conditioned media were collected at various time points, and cytokine levels measured by ELISA. Note that only IL-6 (panel A) and TGF-β1 (panel B) reached detectable and significantly elevated levels as indicated:* $p<0.05$ and ** $p<0.01$.

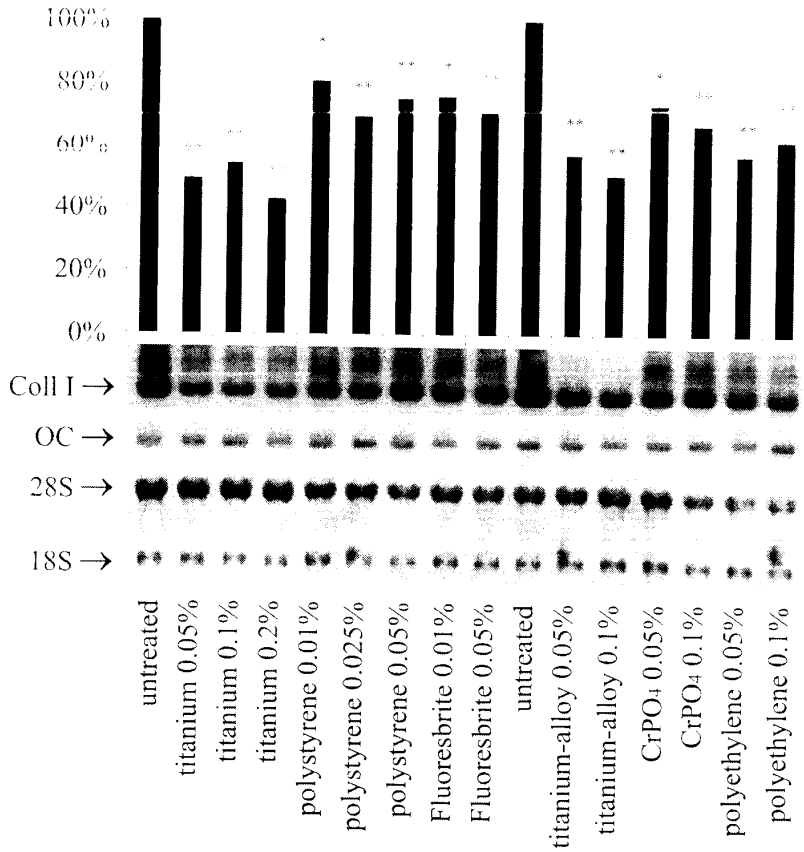

Figure 5.: The effect of various particles on procollagen α1[I] and osteocalcin gene expressions in MG-63 cells. Confluent cell cultures were serum deprived for 24 hours and left either untreated (lanes 1 and 10) or treated with different particles for 48 hours at the various concentrations as indicated. The level of gene expression was compared to untreated samples. Columns represent means of duplicates of at least five independent experiments ± standard deviation of procollagen α1[I] gene expression. Levels of significance are indicated: * $p<0.05$ and ** $p<0.01$. The osteocalcin gene expression in particle-treated MG-63 cells showed no significant differences and a representative hybridization panel (OC) is shown. Note that all types of particles significantly reduced the procollagen α1[I] mRNA expression.

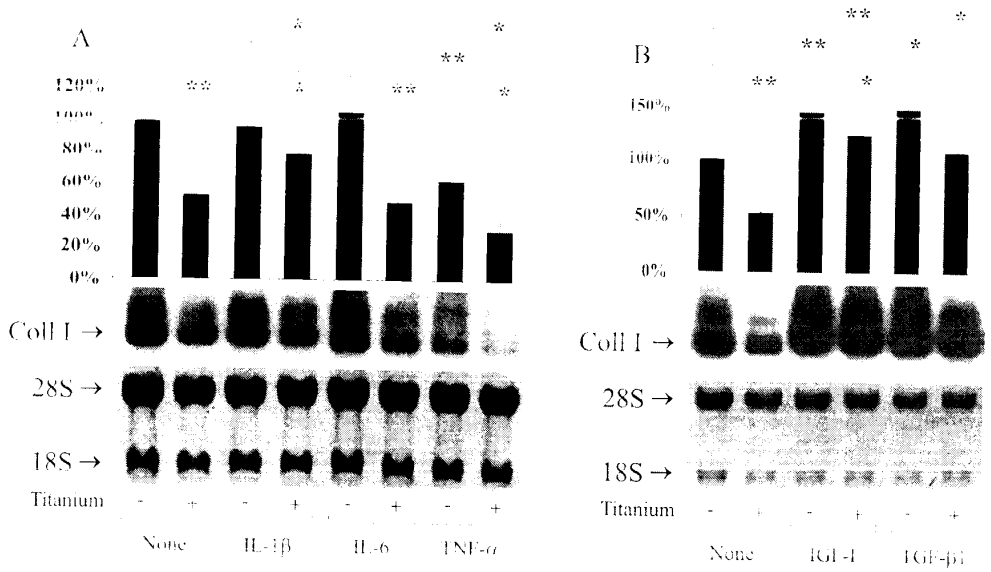

Figure 6.: Northern blot analysis of procollagen α1[I] mRNA expression in MG-63 cells treated with different cytokines (panel A) or growth factors (panel B) in the presence or absence of phagocytosable-size titanium particles. Confluent osteoblast cultures were serum deprived for 24 hours, pretreated with cytokine/growth factor for 3 hours and then titanium particles (0.1 percent, volume/volume) were added where indicated. Bottom panels show the amounts of total (ribosomal) RNA on an ethidium bromide-stained membrane prior to hybridization. Columns represent means of duplicates of at least five independent experiments ± standard deviation. Data are normalized to the mRNA level measured in non-stimulated MG-63 cells (lane 1 on both panels). Levels of significance are indicated: * $p<0.05$ and ** $p<0.01$. Note that neither IL-6 nor IL-1β had an effect on procollagen α1[I] gene expression in particle-free condition, although IL-1β could partially compensate for the suppressive effect of titanium on procollagen α1[I] gene expression.

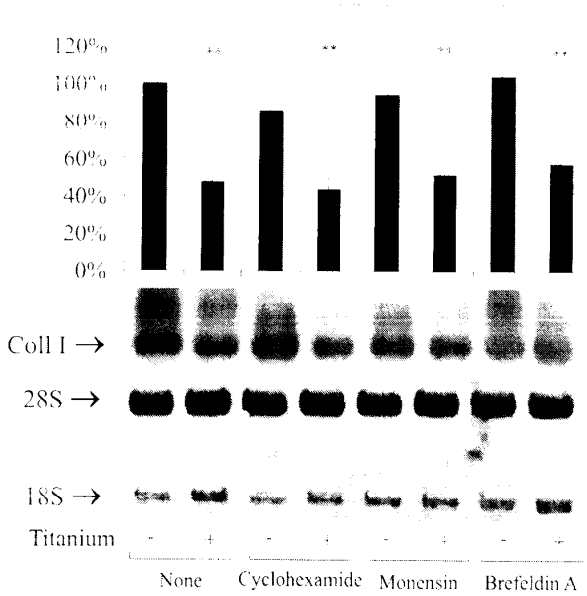

Figure 7.: Effect of protein synthesis and transport inhibitors on procollagen α1[I] gene expression. Confluent MG-63 osteoblast cultures were serum deprived for 24 hours, left untreated or pretreated with the indicated compound for 3 hours and then titanium particles (0.1 percent volume/volume) were added where shown. The level of gene expression was analyzed by Northern blots and compared to untreated samples (lane 1). Columns represent means of duplicates of at least five independent experiments ± standard deviation. Levels of significance are indicated: * $p<0.05$ and ** $p<0.01$. Note that none of the compounds were able to reverse the particle treatment induced gene suppression.

CHAPTER 3

Particulate Wear Debris Activates Protein Tyrosine Kinases and Nuclear Factor-kappa B which Down-regulates Type I Collagen Synthesis in Human Osteoblasts

Csaba Vermes, Kenneth A. Roebuck, Raman Chandrasekaran, József G. Dobai, Joshua J. Jacobs and Tibor T. Glant

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Abstract

Particulate wear debris generated mechanically from prosthetic materials is phagocytosed by a variety of cell types within the periprosthetic space including osteoblasts, which cells with an altered function may contribute to periprosthetic osteolysis. Exposure of osteoblast-like osteosarcoma cells or bone marrow-derived primary osteoblasts to either metallic or polymeric particles of phagocytosable sizes resulted in a marked decrease in the steady-state mRNA levels of procollagen α1[I] and procollagen α1[III]. In contrast, no significant effect was observed for the osteoblast-specific genes, such as osteonectin and osteocalcin. In kinetic studies, particles once phagocytosed, maintained a significant suppressive effect on collagen gene expression and type I collagen synthesis for up to five passages. Large particles of a size that cannot be phagocytosed also downregulated collagen gene expression suggesting that an initial contact between cells and particles can generate gene responsive signals independently of the phagocytosis process. With regard to such signaling, titanium particles rapidly increased protein tyrosine phosphorylation and nuclear transcription factor-kappa B (NF-κB) binding activity prior to the phagocytosis of particles. Protein tyrosine kinase (PTK) inhibitors such as genistein and the NF-κB inhibitor

pyrrolidine dithiocarbamate (PDTC) significantly reduced the suppressive effect of titanium on collagen gene expression suggesting particles suppress collagen gene expression through the NF-κB signaling pathway. These results provide a mechanism by which particulate wear debris can antagonize the transcription of the procollagen α1[I] gene in osteoblasts, which may contribute to reduced bone formation and progressive periprosthetic osteolysis.

Introduction

Particulate wear debris and corrosion products from prosthetic materials can activate a wide variety of cell types within the periprosthetic soft tissue including fibroblasts, osteoclasts, osteoblasts and macrophages. The phagocytosis of particulate wear debris stimulate macrophages to secrete bone-resorbing mediators, including eicosanoids, metalloproteinases and proinflammatory cytokines, such as interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)[1-10]. Fibroblasts of periprosthetic soft tissue (interfacial membrane) constitutively express high levels of metalloproteinases, whereas resting fibroblasts in skin or normal synovial tissue express much lower levels unless exposed to titanium particulates[11-13]. Phagocytosis of particulate biomaterials promotes a signal for osteoclast differentiation and subsequent activation[14,15]. Osteoblasts can also phagocytose particles, which induces IL-6 release and the downregulation of type I collagen synthesis[12,16]. As the particulate wear debris is continuously generated, all these cells at the interface of bone and prosthesis are forced into a constant state of activation[12]. In addition to their direct activation by phagocytosis, there are contributing autocrine and paracrine effects that create a complex milieu within the periprosthetic space, which ultimately governs the development of osteolysis.

Phagocytosis is a non-specific defense mechanism for elimination of tissue debris, foreign particles, bacteria etc., whereby the engulfed material is immediately degraded within

phagosomes. Phagocytosis induces the phosphorylation of tyrosine and/or serine residues of the cytoplasmic tails of phagocytosis receptors. In turn, phagocytosis activates PTKs[17-19] and protein kinase C (PKC)[17,20] pathways, which signaling processes result in altered gene expression via the activation of nuclear transcription factors. The most well characterized *in vitro* cell response to particulate wear debris is the cytokine and prostaglandin release by macrophages and monocytes[3,4,7-11,21,22]. This particulate phagocytosis-induced cytokine release, however, is the consequence of earlier (i.e., more upstream) signaling events.

The aim of this study was to characterize the altered gene responses as a consequence of particulate phagocytosis in bone marrow-derived primary osteoblasts and transformed osteosarcoma cell lines. We studied the effect of phagocytosable small-size (less than 3 μm in diameter) to non-phagocytosable large-size (greater than 20 μm) particles of different compositions (metal and polymeric particles) on osteoblast signaling and gene responses identifying the NF-κB signaling pathway as a major target of the particle effect. These findings indicate that osteoblasts also possess an inflammatory signaling response similar to that of macrophages[1,3]. Particulate wear debris-induced NF-κB activation and IL-6 release accompanied with diminished collagen synthesis may significantly contribute to, and may potentially initiate, the cascade of periprosthetic osteolytic events.

Materials and Methods

Particles

Metal and polymeric particles used in these studies were described previously[1,13,16]. Metal particles of commercially pure titanium with 1-3 μm nominal diameter and chromium-orthophosphate ($CrPO_4x4H_2O$; 1.42 μm ± 0.83)[8] were purchased from Johnson Matthey (Danvers, MA). Titanium 6% aluminum-4% vanadium particles (Sulzer Plasma Technik, Troy, MI) were ground from a 150-300 μm size grinding media[4]. Conventional medical grade

ultra high molecular weight polyethylene base resin (GUR 415, Hoechst-Celanese, Houston, TX) was pulverized by cryogenic attrition in liquid nitrogen and filtrated for size separation[1]. These particles exhibited a comparable size distribution with at least 90% being smaller than three micrometer in diameter. Polystyrene (1.14 um + 0.01) particles were purchased from Polyscience (Warrington, PA). Based upon the size distribution 0.1% (volume/volume: v/v) particle suspension contained approximately 2.2-6.7x10^8 particles/ml. In addition to these small-size particles, large-size titanium particles (21-85 μm) from Johnson Matthey and monodisperse polystyrene particles (21.1 μm ± 4.1) from Polyscience were utilized. Particles were sterilized by irradiation with 2.2 megarad (22,000 gray) from a Cs-137 source (model 143; J.L. Sheppherd Irradiator, San Fernando, CA) and stored in sterile phosphate-buffered saline (PBS; pH 7.2). Endotoxin contamination of particles was excluded by limulus assay (E-Toxate, Sigma Chemical, St. Louis, MO).

Cells and cell cultures

Human osteoblast-like cell lines MG-63, SaOS-2 and HOS were purchased from the American Type Culture Collection (ATCC, Rockville, MD). The cells were cultured in monolayer in Dulbecco's modified Eagle medium (GIBCO, Grand Island, N.Y.) containing 10% fetal bovine serum (FBS; Hyclone Laboratories, Inc., Logan, UT) in a standard tissue culture condition[1,13,16].

Primary osteoblasts were isolated from bone marrow samples of iliac crest bone obtained during spine fusion procedures from patients of either sex, at age range from 25 to 69 years. Collection of bone marrow was approved by the Institutional Review Board and written consent forms were obtained from each patient. Culture conditions, isolation and characterization of cells were exactly the same as described by Mueschler and colleagues[23,24]. Briefly, buffy coat-separated nucleated bone marrow cells (2x10^7/T75 tissue culture flasks;

Corning Inc., Corning, NY) were cultured in alpha-minimal essential medium (Gibco) containing 10% FBS, 10 nM dexamethasone, 50 μg/ml ascorbic acid, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B and 50 μg/ml gentamicin, all purchased from Sigma Chemical Company. The first medium change was performed on day 7, also containing 5 μM beta-glycerophosphate. Semiconfluent cultures were trypsinized, $1x10^5$ cells plated in 10 cm size Petri-dishes (Corning), and cultured to obtain a confluent monolayer culture. All experiments with primary osteoblasts were carried out using these first passage cultures. At the time of the first passage, aliquots of cells were also seeded in 24- and 96-well plates (Corning) for viability tests, cell proliferation and measuring alkaline phosphatase (ALP) activities.

Treatment of cells with particles and various reagents, and sample collection

Confluent cultures of cells were subjected to serum deprivation for 24 h prior to treatment and then the culture media replaced with fresh medium containing 0.3% FBS with particles and/or various compounds at the desired final concentration. Tissue culture media were collected at various time points, centrifuged, filtered through a 0.22 μm polycarbonate filter (Spin-x; Costar, Cambridge, MA) and stored at -80°C. The cell layers were used for RNA, protein and nuclear extractions. All experiments were performed in duplicate or triplicate for at least three independent experiments.

Reagents for targeting specific signal transduction pathways were purchased from Calbiochem (La Jolla, CA) and R&D Systems (Minneapolis, MN). Genistein (20 μM) was used for inhibition of PTK to block the phosphorylation of tyrosine residues. Insulin-like growth factor-I (IGF-I; 30 ng/ml) was used as a PTK activator. H-89 (0.1 μM) was applied to inhibit, and cAMP (10 μM) or dibutyl-cAMP (0.5 mM) to activate cAMP-dependent protein kinase A (PKA). Calphostin C (75 nM) was used to inhibit PKC, while phorbol myristate

acetate (PMA; 25 ng/ml) activates the PKC signaling pathway by binding to PKC. PDTC (100 μM) was applied as an anti-oxidant to block the activation of the NF-κB. Transforming growth factor-beta1 (TGF-β1, 20 pg/ml) was used as a positive control to activate collagen gene expression[25,26]. TNF-α (10 ng/ml), IL-6 (500 pg/ml) and IL-1β (50 pg/ml) were proinflammatory cytokines that increase tyrosine phosphorylation and NF-κB binding activity[27,28]. Cytochalasin D (1 μM) was used to destabilize the cytoskeleton, thus inhibiting phagocytosis. All of the above listed concentrations were selected after serial dilutions (using at least three concentrations) of each compound tested in MG-63 cell cultures.

Viability tests

The trypan blue exclusion was routinely used to determine cell viability[3]. Since the presence of phagocytosed titanium particles, especially at higher concentrations, precluded a precise evaluation of dye-exclusion, cell viability also was determined with fluorescein diacetate (Molecular Probes, Eugene, OR) according to a method previously described[8]. Viability tests were routinely performed in duplicate and at least 200 cells were counted in transmission (for trypan blue exclusion) or epifluorescent mode (for fluorescein diacetate) in a Nikon Microphot-FXA microscope (Nikon, Tokyo, Japan).

RNA extraction and Northern blot hybridization

Total RNA samples were isolated from monolayer cultures as described[13,16]. Approximately 10 μg of total RNA was denatured in 50% formamide, 17.5% formaldehyde and MOPS buffer (20 mM 3-[N-morpholino]propane-sulfonic acid at pH 7.0, 5 mM sodium acetate and 1 mM EDTA at pH 8.0), electrophoresed in 1% agarose gel and transferred to GeneScreen Plus membranes (New England Nuclear, Boston, MA). Membranes were stained with ethidium bromide and the level of 28S and 18S ribosomal RNA quantified by Image1

Software package (Meta Imaging Series, Universal Imaging Corp., West Chester, PA). Blots were hybridized with ^{32}P-dCTP-labeled specific cDNA probes at a concentration of $3x10^6$ cpm/ml (specific activities: $2\text{-}6x10^8$ cpm/μg cDNA)[13]. Human-specific cDNA probes (plasmids) were purchased from ATCC. The following recombinant plasmid DNAs were used as probes: a 1.8 kilobase (kB) long cDNA probe for procollagen α1[I] (Hf677; ATCC #61322), a 1.3 kB cDNA probe for procollagen α1[III] (Hf934; ATCC #61324), a 2.0 kB probe for osteocalcin (ATCC #86269), a 1.8 kB probe for osteonectin (ATCC #78193), a 1.5 kB probe for ALP (ATCC #59633) and a 0.8 kB probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ATCC#57234). Following hybridization, the blots were washed and exposed to Kodak XAR-5 film (Kodak, Rochester, NY) at -70°C or the original Northern blot analyzed by STORM PhosphorImiger using ImigeQuant image analysis software (Molecular Dynamics, Sunnyvale, CA). Probes were frequently paired (e.g. procollagen α1[I] and GAPDH) for hybridization and the same membrane was rehybridized with an another pair of probes (for example, OC and ALP) after stripping the membrane. We found that using different activators and stimulators of various signaling pathways, cytokines, growth factors and their inhibitors, alone and in combination with particle wear debris, the housekeeping genes (for example GAPDH) was occasionally also altered. For this reason, and for comparison of our previous[11,13,16] and current studies, we preferentially used and normalized all Northern hybridization results to the levels of 28S and 18S ribosomal RNA.

Measurement of cytokines and osteoblast-specific proteins in culture media

Cytokine concentrations in supernatants of osteoblast cultures were measured by sandwich enzyme-linked immunosorbent assays (ELISA) in 96-well microtitration plates following the manufacturer's instructions. High sensitivity assay kits for TNF-α (assay range from 0.5 to 32 pg/ml), IL-1β and IL-6 (sensitivity range from 0.2 to 10 pg/ml) and TGF-β1

(range from 31 to 2000 pg/ml) were purchased from R&D Systems. Secreted osteocalcin was measured by NovoCalcin and type I collagen by Procollagen-C ELISAs purchased from Metra Biosystems (Mountain View, CA). ALP activity was measured by Sigma's Alkaline Phosphatase Colorimetric Endpoint assay in cell lysates of primary osteoblasts and confluent cultures stained for ALP positivity using Fast-blue reagent (Sigma) following manufacture's instructions.

Western blot analysis of tyrosine phosphorylation

Treated and untreated cells were lysed in 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 PMSF and 1 U/ml aprotinin), phosphatase inhibitors (50 mM NaH_2PO_4, 10 mM Na-pyrophosphate, 50 mM KF and 1 mM Na_3VO_4) and 0.1% NaN_3 for 1 h at 4°C. Cell lysates were cleared by centrifugation and 15 μg of protein per lane was separated by 10% SDS-PAGE in reducing condition. Samples were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA), and the free binding capacity of the membrane was blocked with 5% skimmed milk in PBS (pH 7.4) for 2 h at room temperature. Phosphorylated tyrosine residues were detected with biotinylated monoclonal anti-phosphotyrosine antibody 4G10 (1:1,000 dilution, Upstate Biotechnology, Lake Placid, NY) followed by Streptavidin-peroxidase (1:4,000 dilution). The reaction was developed by enhanced chemiluminescence (Amersham; Piscataway, NJ).

Electrophoretic mobility shift assays

Transcription factor binding activity was determined by electrophoretic mobility shift assays (EMSA) as described previously[29]. Briefly, nuclear protein extracts (3-6 μg protein) prepared from MG-63 cells and primary osteoblasts by the method of Osborn et al.[30] were

incubated with 50,000 cpm of ^{32}P end-labeled oligonucleotide probes for 20-30 min at room temperature in 10 or 20 µl reaction volumes, containing 12% glycerol, 12 mM HEPES-NaOH (pH 7.9), 60 mM KCl, 5 mM MgCl2, 4 mM Tris-HCl (pH 7.9), 0.6 mM EDTA (pH 7.9), 0.6 mM dithiothreitol, and 0.25 µg of poly-(dI-dC). Oligonucleotide specific for NF-κB (5'-AGTTGAGGGGACTTTCCCAGGC-3') was used to assess binding activity of the nuclear extracts. To demonstrate binding specificity, 100-fold molar excess (10 ng) of the specific or nonspecific (5'-GCAGAGCATATAAGGTGAGGTAGGA-3') oligonucleotides were included in the binding reaction. For supershift analysis, antibodies (1-3 µg) against NF-κB subunits (Rel A, p50, cRel) (Santa Cruz Biotechnology, Santa Cruz, CA) were included in the binding reaction. Protein-DNA complexes were resolved in 5% polyacrylamide gels. Gels were dried and exposed to radiographic film at –70°C.

Statistical analysis

Descriptive statistics were used to determine group means and standard deviations (SD). The Pillai's trace criterion was used to detect multivariate significance. Subsequently, paired Student's "t" tests were performed between groups of interest. Significance levels are indicated in figures. All statistical analyses were performed using personal computer-based statistical software (SPSS/PC+ v 4.0.1, SPSS Inc, Chicago, IL).

Results

Suppression of osteoblast-specific genes by particulate wear debris

We previously reported a consistently 40-60% suppression of procollagen α1[I] mRNA expression in MG-63 osteoblastoid cells after a 48 h exposure to 0.1% titanium particles (1-3 µm)[16]. To further characterize the particle effect on osteoblast-specific gene expression and protein synthesis, MG-63, SaOS-2 and HOS osteosarcoma cells and bone

marrow-derived primary osteoblasts were exposed for 48 h to various metallic and polymeric particles including titanium, titanium alloy, chromium orthophosphate, polystyrene and polyethylene. As shown in Fig. 1, particles, regardless of type, suppressed steady state levels of procollagen α1[I] and α1[III] mRNA expression in both MG-63 osteosarcoma cells and in bone marrow-derived primary osteoblasts. This downregulation of collagen gene expression was accompanied by reduced type I collagen synthesis (Figs. 2 and 3B). In contrast to the affects on procollagen expression, the various particles did not alter significantly the expression of osteocalcin (Fig. 1), or other osteoblast-specific genes such as osteonectin and ALP (data not shown). A similar particle effect was also observed in SaOS-2 and HOS osteoblastoid cell lines (data not shown) representing that this phenomenon was not limited to one cell line. These data demonstrate that particles differentially affect gene expression in osteoblasts and indicate that the gene-specific effects are in response to particulates in general and not to a specific type of particle or cell type.

To determine if phagocytosis of the particle was critical for the suppression of collagen gene expression, we assessed the effects of the cytoskeleton inhibitor cytochalasin D, which interferes with the phagocytosis process. Pretreatment of the cells with cytochalasin D markedly reversed, albeit not completely, the titanium-induced suppression of procollagen α1[I] gene expression. To further assess the role of phagocytosis, we exposed the cells to large particles that were too large to be phagocytosed by osteoblasts (Fig. 1). Although large particles suppressed collagen gene expression significantly, this effect was modest compared to the suppression produced by small particles. Together, these data suggest that although phagocytosis clearly plays the major role in the suppression of collagen gene expression, the particle effect appears to also be mediated, at least in part, by a phagocytosis independent mechanism. Thus, particle interactions with the cell prior to phagocytosis of the particle can likely also elicit gene responsive signals.

The long-term effect of phagocytosed particles

To assess the long-term effects of phagocytosed particles on osteoblast function, MG-63 cells were exposed to small-size titanium particles for 72 h and either harvested for RNA isolation, or passaged in standard conditions containing 10% FBS without further particle treatment. The medium and newly passaged MG-63 cells were harvested after 48 h, and an aliquot of cells was passaged again. Procollagen α1[I] mRNA expression was assessed by Northern blot analysis and secreted type I collagen measured by ELISA at each 48 h time point. As shown in Fig. 2, the initially suppressed procollagen α1[I] mRNA gradually increased from passage to passage and reached a level close to normal after 5 passages, i.e. 13 days after the particle exposure. Secretion of type I collagen, while delayed, essentially paralleled with mRNA levels (Fig. 2), except after the first passage, after which cells were cultured again in 10% FBS-containing medium. This relatively long recovery period, even under optimal culture conditions, suggests that few particles, retained intracellularly (confirmed by microscopic evaluation), can continue to suppress type I collagen expression and synthesis.

Cytokine release by osteoblasts and the effect of exogenous cytokines upon procollagen α1[I] gene expression

To further characterize the effects of particles on osteoblast function, we next examined cytokine release from particle-treated cells. The earliest cytokine release that could be detected in culture medium was 12 h. However, of the cytokines and growth factors examined, the only cytokine we were able to detect was IL-6 (Fig. 3A) with primary osteoblasts secreting approximately three times more IL-6 than the MG-63 cells (data not shown). In contrast, no IL 1β, TGF β1 or TNF-α was detected in the culture medium even after 24-h of culture (data not shown).

As TNF-α is a known NF-κB activator, this proinflammatory cytokine increases IL-6 release and suppresses procollagen gene expression and type I synthesis via NF-κB activation[31,32], this cytokine was used as a positive control in most experiments. However, MG-63 cells were treated also with various cytokines (IL-1β, IL-6) and growth factors (TGF-β1, IGF-I). As a summary of these experiments, only TNF-α had a significant effect on IL-6 secretion by MG-63 cells (Fig 3A). Similarly, only TNF-α had a strong effect on type I procollagen expression suppressing collagen synthesis even better than titanium particles (Fig 3B). When titanium particles and TNF-α were added together the suppression was even greater, in both MG-63 and primary osteoblasts, and their effect seemed to be additive but not synergistic (manuscript in preparation). The TNF-α suppression of collagen is consistent with previous reports in other cell types[33-35]. While increased IL-6 secretion correlated inversely with procollagen α1[I] expression, neither exogenous IL-6 nor neutralizing antibodies to IL-6 altered the collagen gene expression (data not shown). In contrast to TNF-α and titanium particles, IGF-I and TGF-β1, which had no effect on the secretion of other cytokines in any osteoblast cultures tested, markedly increased collagen gene expression and could completely reverse the titanium-induced suppression of procollagen α1[I] mRNA (data not shown).

Particle-induced signaling in osteoblasts

Because particle-cell interactions appeared to initiate gene responses prior to particle engulfment, we next investigated whether osteoblasts exposed to titanium particles could rapidly stimulate intracellular signaling events and activate nuclear transcription factors. Because tyrosine phosphorylation is an early signaling response in the activation of several inducible transcription factors such as activator protein-1 (AP-1) and NF-κB, we first assessed the effect of titanium particles on PTK activity. To directly demonstrate that

titanium particles activate PTK activity in osteoblasts, MG-63 cells were exposed to titanium particles for 10, 30, 60, and 120 min and tyrosine-phosphorylated proteins were detected by Western blotting using an anti-phosphotyrosine antibody (4G10). As shown in Fig. 4, titanium particles increased the tyrosine phosphorylation of a number of proteins by 120 min. More importantly, tyrosine phosphorylation of at least one protein (with approximately 32 kDa) could be detected as early as 10-30 min (Fig. 4.) demonstrating that osteoblasts exposed to titanium particles can elicit an activation signal well before phagocytosis of the particle.

Many transcription factors can be activated via PTK, including NF-κB[27,36]. Titanium particles induced two specific NF-κB binding complexes within 2-3 h of particle exposure in either MG-63 (Fig.5A) or primary osteoblasts (Fig. 5B). The activation of NF-κB was relatively specific since titanium particles did not activate other transcription factors, such as AP-1 or Sp1 (data not shown). Consistent with the gene responses, large-size particles also activated NF-κB (Fig. 5A, lane 10) demonstrating that particle-cell interactions can activate signal transduction independently of phagocytosis.

To identify the NF-κB subunit composition of the NF-κB gel shift complexes induced by titanium particles, we performed a supershift analysis using antibodies against NF-κB1 (p50), Rel A (p65) and cRel. As shown in Fig. 5, both anti-Rel A and anti-NF-κB1 but not anti-cRel antibodies, produced supershifts indicating that the NF-κB binding complexes are composed of either NF-κB1 and Rel A homodimers or NF-κB1/Rel A heterodimers. Collectively, the DNA binding studies indicate that titanium particles can activate the NF-κB signaling pathway in osteoblasts providing a direct mechanism by which particulate wear debris can generate specific gene responses.

Particle suppression of collagen gene expression is dependent on the NF-κB signaling pathway

To determine whether the activation of PTK pathway and NF-κB was necessary for (titanium) particle-induced gene suppression, we assessed the effects of PTK and NF-κB inhibitors on procollagen α1[I] gene expression and NF-κB activation. PTK inhibitors genistein (Fig. 6) and herbimycin A (data not shown), and NF-κB inhibitors PDTC (Fig. 6) and TPCK (data not shown) essentially abolished the suppressive effect of titanium particles. More clearly, neither NF-κB activation nor procollagen α1[I[mRNA suppression were detected when MG-63 or primary osteoblast cells were pre- and co-treated with either genistein or PDTC during particle treatment. These data suggest not only a correlation but also a direct linkage between tyrosine phosphorylation, NF-κB activation, and procollagen α1[I] gene suppression in titanium-challenged osteoblasts.

In contrast to PTK and NF-κB inhibitors, the inhibition of cAMP-dependent PKA either increased or did not alter the procollagen α1[I] gene expression, and had absolutely no effect upon titanium-induced suppression of the gene (Fig. 6). Like PKA, the PKC pathway also appears not to be involved in collagen gene suppression since the PKC activator PMA and the PKC inhibitor calphostin C had no significant effect on titanium particle treatment-induced gene suppression (Fig. 6). As PMA is a potent NF-κB activator via PKC in various cell types, we also tested the PMA effect on NF-κB activation in MG-63 cells. PMA, in a wide range of concentration (from nanomolar to micromolar), exhibited only a very modest effect upon NF-κB activation, which was at least 5-10-fold less than found in either titanium particle- or TNF-α–stimulated MG-63 cells. Furthermore, the PMA-induced subunit composition of the NF-κB complex seemed to be different by EMSA than shown in Fig. 6. (manuscript in preparation). In summary, PMA-induced NF-κB activation did not influence

procollagen α1[I] gene expression likely due to the insufficient level of activation and/or to the activation of specific complexes which were distinct from the complexes activated by either titanium or TNF-α through PTKs. In conclusion, we find a striking correlation between suppression of procollagen α1[I] gene expression and inhibition of the NF-κB signaling pathway.

Discussion

In this study, we investigated the specific gene responses induced in osteoblasts exposed to particulate wear debris. We demonstrated that particles, regardless of their type, can downregulate collagen gene expression in either human osteosarcoma cell lines or bone marrow-derived primary osteoblasts. The particle effect in osteoblasts was gene-specific since particles had little or no effect on several osteoblast-specific genes including osteonectin and osteocalcin. This differential gene expression in response to phagocytosed particles indicates that particle-cell interactions activate specific signaling events and transcription factors in osteoblasts leading to the upregulation of IL-6 and downregulation of procollagen α1[I]. Indeed, we found that particles rapidly activate protein tyrosine phosphorylation and induce the nuclear transcription factor NF-κB. The rapid kinetics of the activation suggest the particles can elicit signals prior to the phagocytosis process. Along these lines, we demonstrated that particles too large to be phagocytosed could also suppress collagen gene expression though to a lesser degree. Consistent with these data large-size titanium particles can also activate NF-κB. Inhibition of NF-κB function by either tyrosine kinase inhibitors or anti-oxidants reversed the suppressive effect of titanium particles on procollagen α1[I] gene expression suggesting a functional relationship in osteoblasts between tyrosine phosphorylation, NF-κB activation, and collagen gene expression. Thus, particle-cell interactions prior to their phagocytosis appear to initiate an intracellular tyrosine

phosphorylation cascade that targets the nuclear activation of the inducible transcription factor NF-κB.

The transcription factor NF-κB is activated by a wide variety of extracellular stimuli and has emerged as a key regulator of the inflammatory response[28,37-39]. In the DNA binding studies, two induced gel shift complexes were detected, suggesting titanium particles activate at least two NF-κB subunits. Supershift analysis identified Rel A and NF-κB1 (p50) as subunit components of the induced NF-κB binding complexes. Although NF-κB generally activates gene transcription, titanium particle induction of NF-κB in osteoblasts paradoxically leads to the suppression of collagen gene expression. However, TNF-α, one of the most potent activators of the NF-κB pathway, also leads to the suppression of collagen in osteoblasts as well as other cell types[33-35]. Recently, it was shown that NF-κB activation was involved in the TNF-α suppression of procollagen α2[I] mRNA expression in fibroblasts[31] demonstrating that NF-κB interactions with the promoter could mediate downregulation of collagen. In that study, a TNF-α responsive element that binds NF-κB was localized to a regulatory sequence in the proximal promoter of the procollagen α2[I] gene. We recently found that the procollagen α1[I] gene also contains similar, variant NF-κB binding site which also bind Rel A and NF-κB1 (p50) subunits (manuscript in preparation). NF-κB1 lacks an activation domain and homodimers of NF-κB1 have been reported to act as a negative transcription factor capable of downregulating gene expression from NF-κB binding sites[40]. It is possible that titanium downregulates procollagen α1[I] gene expression via these upstream NF-κB binding sites in a manner analogous to the TNF-α downregulation of procollagen α2[I] gene expression. Indeed, recently it was shown that the murine procollagen α1[I] gene is downregulated by Rel A subunit of NF-κB via an interaction with the transcription factor Sp1[32]. Interestingly, the human procollagen α1[I] gene promoter also contains Sp1 binding

sites[41-44] suggesting the mouse and human genes may be downregulated through similar mechanisms.

One of the most important observations of this study was that altered osteoblast function could be achieved by either an initial and early particulate-cell interaction (large-size particles without phagocytosis) or the long-term presence of previously phagocytosed particles. Thus, particles can initiate gene responses in osteoblasts independently of phagocytosis, although a long-term cell response appears to require particle phagocytosis. In support of a direct role of phagocytosis, we found that cytochalasin D, which disrupts actin cytoskeleton filaments critical for the phagocytosis process, significantly reduced the suppression of procollagen α1[I] gene expression in osteoblasts. Phagocytosis in macrophages, which are professional phagocytic cells[10,45], activates NF-κB. However, other cells (e.g., fibroblasts and osteoclasts) can also phagocytose non-degradable particles[13-15,45] forcing them into a chronic state of activation. Thus, particulate wear debris can directly activate osteoclasts, and dramatically alter osteoblast function by both inducing IL-6 secretion and suppressing collagen synthesis. Consequently osteoblast-secreted IL-6 can further activate osteoclasts[46-48]. Clinically, this chronic cell activation may upset the delicate balance between bone formation (diminished osteoblast function) and bone resorption (activated osteoclast function) leading to periprosthetic osteolysis.

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Figures and figure legends

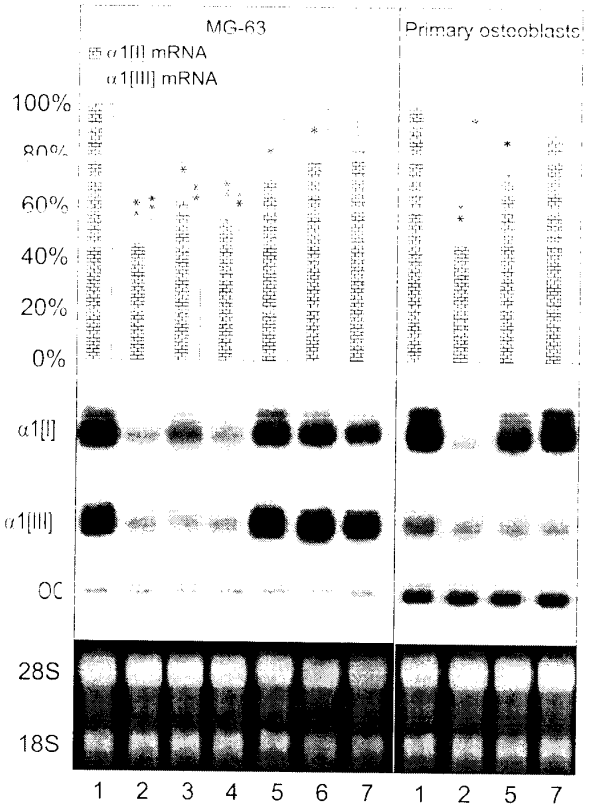

Figure 1.: Effect of particles on procollagen α1[I], α1[III] and osteocalcin mRNA expression in osteoblasts. Confluent monolayer cultures of MG-63 cells (left side panel) and bone-marrow-derived primary osteoblasts (right side panel) were serum deprived, exposed to particles for 48 h, and gene-specific mRNAs detected by Northern blot hybridization. Bottom of figure shows the amounts of total RNA on ethidium bromide-stained membranes prior to hybridization. The membrane was hybridized with osteocalcin, type III and then type I collagen cDNA probes as described in Methods. Osteoblasts were untreated (lane 1) or treated with 0.1% titanium (lane 2), 0.1% polystyrene (lane 3), 0.1% chromium orthophosphate (lane 4). In lane 5, cells were treated with cytochalasin D 1 μM for 3 h followed by 0.1% titanium (1-3 μm). In lane 6, MG-63 cells were treated with large-size polystyrene and in lane 7 ,both MG-63 and primary osteoblasts with large-size titanium particles (grater than 20 μm). Columns at the top of the figure represent means of duplicates of at least three independent experiments + SD. Levels of significance are indicated: * $p<0.05$ and ** $p<0.01$.

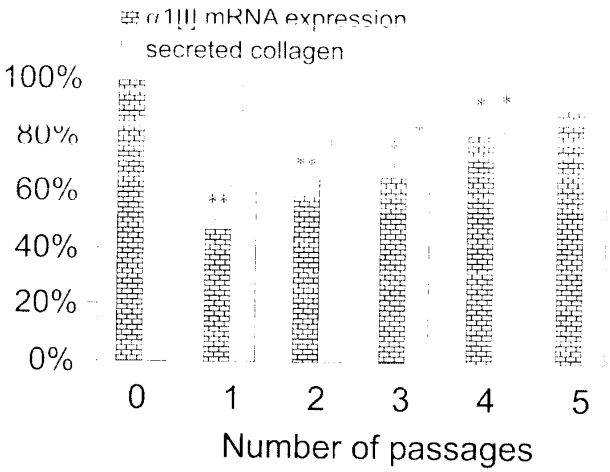

Figure 2.: Long term effect of particles on procollagen α1[I] mRNA expression and type I collagen secretion in MG-63 cells. MG-63 cells were either untreated (0) or initially treated with 0.1% titanium (1-3 μm) for 72 h (1) and then passaged in every 48 h. Aliquots of cells were harvested for RNA extraction and culture media for ELISA at the time of each passage. Numbers represent passage numbers (2-5). The mRNA level was normalized to total RNA and secreted type I collagen (measured as type I procollagen-C) to cell number of identically cultured but untreated MG-63 cells. Data represent the mean of duplicates of at least three independent experiments ± SD. Levels of significance, relative to untreated samples, are indicated: *$p<0.05$, **$p<0.01$.

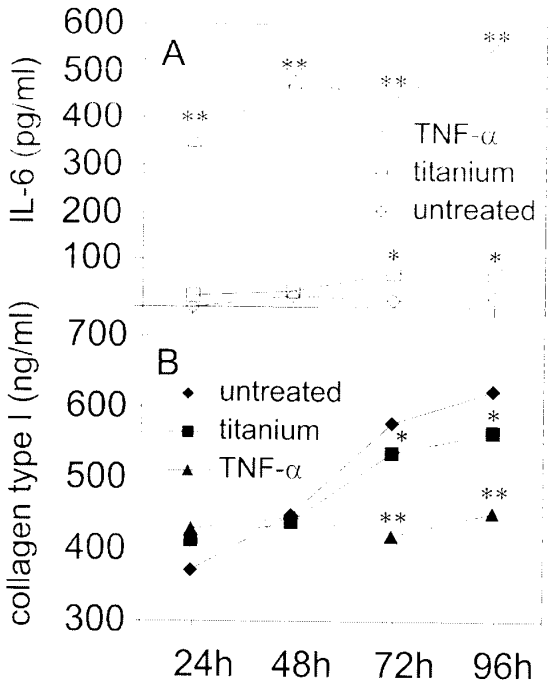

Figure 3.: IL-6 (A) and type I collagen (B) production by MG-63 cells in the presence or absence of 0.1% titanium particles (1-3 μm) or exogenous TNF-α (10 ng/ml). Conditioned media were collected at various time points and both collagen (as type I procollagen-C) and IL-6 were measured by ELISA. Levels of significance, relative to untreated cultures, are indicated: * $p<0.05$ and ** $p<0.01$. Error bars are omitted for clarity.

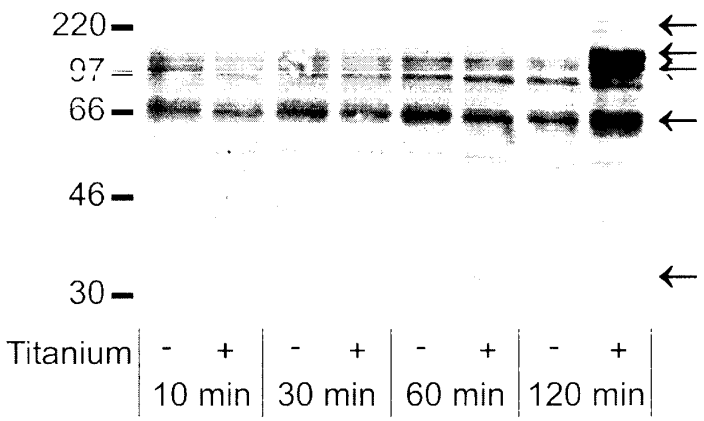

Figure 4.: Effect of titanium particles on protein tyrosine phosphorylation in MG-63 osteoblasts. Cells were exposed to 0.1% titanium particles (1-3 μm) for various time periods. Samples were prepared as described in Methods. Arrows show titanium-induced protein tyrosine phosphorylation at different time points as indicated. Molecular weight standards in kDa are specified on left.

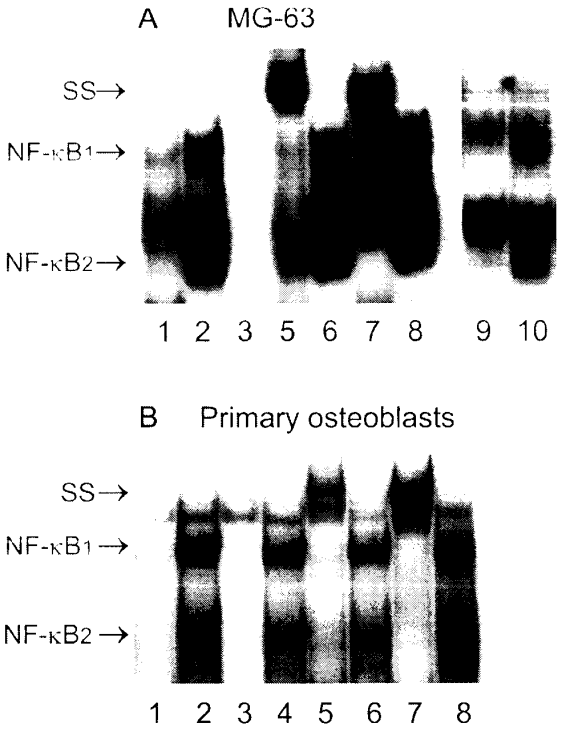

Figure 5.: EMSA and supershift analysis of NF-κB binding activity in osteoblasts. MG-63 (A) and primary human osteoblast (B) cells were untreated (lanes 1 and 9) or treated with small-size 0.1% titanium particles (lane 2), large-size titanium particles (lane 10) or 10 ng/ml TNF-α (lane 8) for 2 h. Arrows indicate the titanium-induced NF-κB binding complexes (NF-κB1 and NF-κB2). In competitive systems, specific (lane 3) and non-specific (lane 4) unlabeled oligonucleotides were mixed in 100-fold molar excess with nuclear extracts from TNF-α-stimulated cells to demonstrate that the induced NF-κB complexes were specific. For supershift (SS) analysis, the presence of p50 and Rel A (lanes 5 and 7) but not cRel (lane 6) was detected by subunit specific antibodies. The free unbound oligonucleotide probe that migrates to the bottom of the gel is not shown.

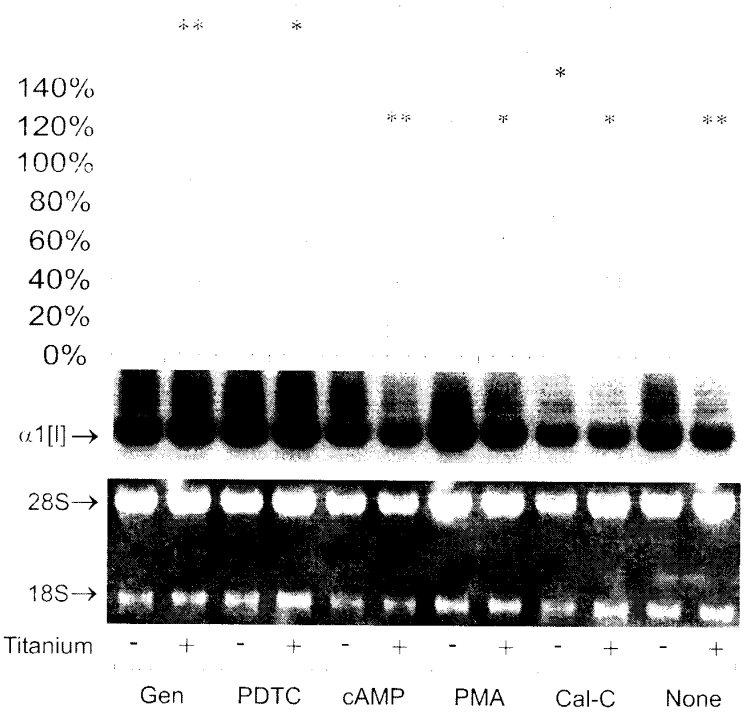

Figure 6.: Effect of inhibitors/activators of various signaling pathways on procollagen α1[I] mRNA expression in MG-63 cells. Confluent cultures of osteoblasts were serum deprived for 24 h, pretreated with the indicated compound for 3 h. Titanium particles (0.1%, 1-3 μm) were then added to the pretreated cultures for an additional 48 h. The last two columns show the procollagen α1[I] mRNA expression in MG-63 cells either untreated (lane 11) or treated with 0.1% titanium (lane 12) without any additional agent. Other lanes represent treatments with genistein (Gen), PDTC, cAMP, PMA and calphostin C (Cal-C) in the presence or absence of 0.1% titanium particles as indicated. Middle panel shows a representative autoradiograph of Northern hybridization with the corresponding total RNA (bottom panel) on ethidium bromide-stained membrane prior to hybridization. Columns represent means of duplicates of at least three independent experiments ± SD. Levels of significance are indicated: * $p<0.05$ and ** $p<0.01$. Note that only PTK inhibitor genistein (lane 2) and NF-κB inhibitor PDTC (lane 4) were able to reverse titanium-induced procollagen α1[I] gene suppression.

CHAPTER 4

SUMMARY

Discussion

While the joint replacement procedure has provided dramatic pain relief and improvement of quality of life for millions of people with end-stage joint disease, the problem of these patients may only be solved temporarily. Artificial joints, even of the most optimal metal and polymeric materials, have initiated a unique pathological condition. With the implantation of these artificial materials, we expose patients to a new biological problem. Billions of ultra fine non-degradable particles are generated *in-vivo*, which accumulate adjacent to the implant and bone, eliciting chronic tissue reaction leading to osteolysis in the bone structure surrounding the implant. Despite the success of joint replacements, aseptic loosening frequently associated with periprosthetic osteolysis jeopardizes the long-term success of both cemented and cementless total hip replacements.

Bone is a dynamic tissue and its' homeostasis is preserved by coupled bone formation and resorption. However, in pathological situations the balance between formation and resorption could be shifted to the latter resulting in bone loss. Such a state exists in the periprosthetic milieu in the presence of implant-derived corrosion products and wear debris. Osteoclasts and osteoblasts phagocytose particles *in-vitro*, and it is assumed that this occurs *in-vivo* as well. During particle phagocytosis, osteoblasts become activated and produce IL-6[1-4], IL-8[5], MCP-1[5] and PGE2[6]. Simultaneously, osteoblasts loose their capacity to synthesize type I collagen in the presence of particulate wear debris[1,2,7,8], because of the suppression of procollagen α1[I] gene transcription mediated by NF-κB activation. The secreted IL-6[9] and PGE2[10] then activate osteoclasts in a paracrine fashion. Other cytokines such as IL-1β and TNF-α are also present in the periprosthetic tissue. TNF-α can activate osteoblasts to secrete IL-6 and suppress type I collagen synthesis and proliferation[1,2]. In addition, both IL-1β and

TNF-α induce osteoclast differentiation from precursor cells and activate differentiated osteoclasts *in-vitro*[10]. Taken together, phagocytosis directly, and phagocytosis-induced cytokine release indirectly, negatively affect bone turnover via alteration of osteoblast and osteoclast functions.

Among a number of cytokines and growth factors, IGF I and TGF β1 were able to completely reverse the suppressive effect of particles on procollagen α1[I] gene expression[2]. These growth factors, when used alone, significantly up-regulated the procollagen α1[I] gene expression[2] and type I collagen synthesis. Furthermore, these growth factors increased osteoblast proliferation without affecting cell viability or inducing substantial IL-6 secretion[2]. Thus, IGF-I and TGF-β1 might be potent inducers of bone matrix formation even in the presence of wear debris. Likewise, compounds inhibiting intracellular signaling mechanisms (PTK-NF-κB) of the osteoblast triggered by particulate wear debris and cytokines had the ability to normalize many of the altered functions of human osteoblasts[1].

Eventually, local delivery of certain growth factors (e. g. IGF-I or TGF-β1)[2], protein tyrosine kinase inhibitors (e. g. Genistein) or NF-κB inhibitors (e. g. PDTC)[1,8] (all of which can reverse the suppressive effect of either proinflammatory cytokines or wear particles on osteoblast functions) may have a therapeutic role in the prevention and/or treatment of periprosthetic bone loss.

Significance

We hypothesized that osteoblast may play an important role in the etiopathogenesis of periprosthetic osteolysis. In these studies, our hypothesis has been proved by the data we have gained following having summarized the effects of the components of the periprosthetic milieu on the functions of human osteoblasts. Furthermore, we have specified intracellular signaling mechanisms by which these factors mediate their impact on osteoblasts. The results we have gathered thorough this study, presented in Chapter 2 and Chapter 3, are considered

to be unique and novel in this research field. According to this, we can conclude that degradation products from implant materials directly and soluble factors released by other periimplant cells via a paracrine manner negatively influences the functions of human osteoblasts. These cells loose their capacity to synthesize components of the extracellular bone matrix and simultaneously produce osteoclast activating factors resulting in a significant shift of the bone metabolism to bone resorption in the periprosthetic space.

One of the most important findings of this study is that, besides the detailed exploration of a highly unique bone cell behavior, this study provides not only the description of pathomechanism governing periimplant osteolysis, but also points out molecules that could be potential targets for pharmacological intervention of aseptic implant loosening by restoring the functions of the osteoblast in the periprosthetic area.

We believe that this work contains significant information in this field, provides a deeper insight into the complexity of periprosthetic osteolysis and contributes to the development of feasible therapeutic approaches for aseptic implant loosening in the future.

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PODIUM AND POSTER PRESENTATIONS

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40th Annual Meeting of Hungarian Orthopaedic Society, 1997, Szekszárd, Hungary

2. **Vermes, C.**, Than, P., Bálint, L.: The operative treatment of cystic bone disorders adjecent to the articular surface using allogenous bone from bone bank
Forum of Young Hungarian Orthopaedic Surgeons, 1997, Agárd, Hungary

3. **Vermes, C.**, Than, P., Bálint, L.: Bilateral cystic bone disorders of the foot. Report of two cases
2nd Central European Orthopaedic Meeting, 1998, Budapest, Hungary

4. Bellyei, Á., Than, P., **Vermes, C.**: The possibilities of prosthesis implantation after osteotomies around the hip
2nd Central European Orthopaedic Meeting, 1998, Budapest, Hungary

5. Dobai, J., Chandrasekaran, R., Narayanan, R., **Vermes, C.**, Andersson, G.B.J., An, H., Jacobs, J.J., Mueschler, G.F., Boehm, C., Carpenter, L., Roebuck, K.A., Glant, T.T.: Suppressed collagen gene expression and diminished collagen synthesis induced by particulate wear debris in bone marrow-derived osteoblasts are reversed by 1,25(OH)$_2$D$_3$ **(poster)**
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63rd Annual Scientific Meeting, American College of Rheumatology, 1999, Boston, Massachusetts, USA

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46th Annual Meeting, Orthopedic Research Society, 2000, Orlando, Florida, USA

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6th World Biomaterials Congress, 2000, Kamuela, Hawaii, USA

11. Than, P., **Vermes, C.**, Schäffer, B., Lőrinczy, D.: Differential Scanning Calorimetric examination of the human hyaline cartilage
3rd Central European Orthopedic Meeting, 2000, Portoroz, Slovenia

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64th Annual Scientific Meeting, American College of Rheumatology, 2000, Philadelphia, Pennsylvania, USA

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47th Annual Meeting, Orthopedic Research Society, 2001, San Francisco, California, USA

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1st annual William H. Harris Lectureship, 2001, Boston, Massachusetts, USA

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Michigan State University Affiliated Orthopedic Basic Science Seminar, 2001, Grand Rapids, Michigan, USA

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Rush University Forum for Research and Clinical Investigation, 2001, Chicago, Illinois, USA

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Rush University Forum for Research and Clinical Investigation, 2001, Chicago, Illinois, USA

20. Hallab, N.J., Mikecz, K., **Vermes, C.**, Skipor, A., Glant T.T.: Human lymphocyte reactivity to serum derived metal protein complexes **(poster)**
Rush University Forum for Research and Clinical Investigation, 2001, Chicago, Illinois, USA

21. Firneisz, G., Hanyecz, A., **Vermes, C.**, Bárdos, T., Glant, T.T.: Gene expression profile in an animal model of rheumatoid arthritis using cDNA microarrays **(poster)**
65th Annual Scientific Meeting, American College of Rheumatology, 2001, San Francisco, California, USA

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65th Annual Scientific Meeting, American College of Rheumatology, 2001, San Francisco, California, USA

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65th Annual Scientific Meeting, American College of Rheumatology, 2001, San Francisco, California, USA

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48th Annual Meeting, Orthopedic Research Society, 2002, Dallas, Texas, USA

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Rush University Forum for Research and Clinical Investigation, 2002, Chicago, Illinois, USA

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66th Annual Scientific Meeting, American College of Rheumatology, 2002, New Orleans, Lousiana, USA

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29th Annual Meeting of Society For Biomaterials, 2003, Reno, Nevada, USA

44. Kustos, T., Bellyei, Á., **Vermes, C.**: Long term results of subcutaneous versus open tenotomy of the calcaneal tendon in children suffering from ICP
46th Annual Meeting of Hungarian Orthopaedic Society, 2003, Budapest, Hungary

45. Firneisz, G., Zehavi, I., **Vermes, C.**, Hanyecz, A., Frieman, J.A., Glant, T.T.: A novel combination of methods to identify and quantify disease-related gene clusters **(poster)**
66th Annual Scientific Meeting, American College of Rheumatology, 2003, San Fransisco, California, USA