ADAMs, OSTEOCLASTOGENESIS AND BONE DESTRUCTION IN THE LOOSENING OF THE TOTALLY REPLACED HIP

GuoFeng Ma

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1. LIST OF ORIGINAL PUBLICATIONS


IV. Ma GF, Porola P, Hedman K, Salo J, Konttinen YT: ADAM8 expression in parainfluenza virus 2 (PIV2) induced human salivary adenocarcinoma cell line (HSY) cells fusion or the formation of multinuclear giant cells, BMC Microbiology (submitted).
### 2. ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>avidin-biotin-peroxidase complex</td>
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<tr>
<td>ADAM</td>
<td>a disintegrin and a metalloproteinase</td>
</tr>
<tr>
<td>ADAMTSs</td>
<td>a disintegrin and metalloproteinase with thrombospondin motifs</td>
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<tr>
<td>ARF</td>
<td>activation-resorption-formation</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine triphosphatase</td>
</tr>
<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolylphosphate</td>
</tr>
<tr>
<td>BMU</td>
<td>basic multicellular unit</td>
</tr>
<tr>
<td>DAB</td>
<td>3, 3'-diaminobenzidine tetrahydrochloride</td>
</tr>
<tr>
<td>DAPI</td>
<td>4', 6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>FDA</td>
<td>United States Food and Drug Administration</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
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<tr>
<td>GMK</td>
<td>green monkey kidney (cell line)</td>
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<tr>
<td>HSY</td>
<td>human salivary adenocarcinoma (cell line)</td>
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<td>IFN-γ</td>
<td>interferon-γ</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>ISH</td>
<td><em>In situ</em> hybridization</td>
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<tr>
<td>M-CSF</td>
<td>macrophage-colony stimulating factor</td>
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<tr>
<td>MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<tr>
<td>NBT</td>
<td>nitroblue tetrazolium</td>
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<tr>
<td>OPG</td>
<td>osteoprotegerin</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cell</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PIV-2</td>
<td>Parainfluenza virus type 2</td>
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<tr>
<td>PMMA</td>
<td>polymethylmethacrylate</td>
</tr>
<tr>
<td>PGE</td>
<td>prostaglandin</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
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<tr>
<td>RANK</td>
<td>receptor activator of nuclear factor kappa B</td>
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<td>RANKL</td>
<td>receptor activator of nuclear factor kappa B ligand</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>SH3</td>
<td>Src homology 3</td>
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<tr>
<td>SR</td>
<td>scavenger receptor</td>
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<tr>
<td>TGF-α</td>
<td>transforming growth factor-α</td>
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<td>THR</td>
<td>total hip replacement</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<td>UHMWPE</td>
<td>ultrahigh molecular weight polyethylene</td>
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3. ABSTRACT

Total hip replacement is the golden standard treatment for severe osteoarthritis refractory for conservative treatment. Aseptic loosening and osteolysis are the major long-term complications after total hip replacement. Foreign body giant cells and osteoclasts are locally formed around aseptically loosening implants from precursor cells by cell fusion. When the foreign body response is fully developed, it mediates inflammatory and destructive host responses, such as collagen degradation. In the present study, it was hypothesized that the wear debris and foreign body inflammation are the forces driving local osteoclast formation, peri-implant bone resorption and enhanced tissue remodeling. Therefore the object was to characterize the eventual expression and the role of fusion molecules, ADAMs (an abbreviation for A Disintegrin And Metalloproteinase, ADAM9 and ADAM12) in the fusion of progenitor cells into multinuclear giant cells. For generation of such cells, activated macrophages trying to respond to foreign debris play an important role. Matured osteoclasts together with activated macrophages mediate bone destruction by secreting protons and proteinases, including matrix metalloproteinases (MMPs) and cathepsin K. Thus this study also assessed collagen degradation and its relationship to some of the key collagenolytic proteinases in the aggressive synovial membrane-like interface tissue around aseptically loosened hip replacement implants.

ADAMs were found in the interface tissues of revision total hip replacement patients. Increased expression of ADAMs at both transcriptional and translational levels was found in synovial membrane-like interface tissue of revision total hip replacement (THR) samples compared with that in primary THR samples. These studies also demonstrate that multinucleate cell formation from monocytes by stimulation with macrophage-colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL) is characterized by time dependent changes of the proportion of ADAMs positive cells. This was observed both in the interface membrane in patients and in two different in vitro models. In addition to an already established MCS-F and RANKL driven model, a new virally (parainfluenza 2) driven model (of human salivary adenocarcinoma (HSY) cells or green monkey kidney (GMK) cells) was developed to study various fusion molecules and their role in cell fusion in general. In interface membranes, collagen was highly degraded and collagen degradation significantly correlated with the number of local cells containing collagenolytic enzymes, particularly cathepsin K.

As a conclusion, fusion molecules ADAM9 and ADAM12 seem to be dynamically involved in cell-cell fusion processes and multinucleate cell formation. The highly significant correlation between collagen degradation and collagenolytic enzymes, particularly cathepsin K, indicates that the local acidity of the interface membrane in the pathologic bone and soft tissue destruction. This study provides profound knowledge about cell fusion and mechanism responsible for aseptic loosening as well as increases knowledge helpful for prevention and treatment.
4. INTRODUCTION

In aseptic loosening, foreign body reaction is directed against microparticles that formed by corrosion and micromovement between implant and bone. Peri-implant osteolysis develops as a consequence of an active biologic process involving the resorption of bone at the peri-implant sites and leads to loss of prosthetic fixation, dissection of the implant and extension of the effective joint space. The macrophages, foreign body giant cells, and osteoclasts that are present within the peri-implant tissues are derived from a common hematopoietic lineage, but their phenotypic genes, gene products and function are different to distinguish these cells from each other (Shen et al. 2006). Foreign body giant cells recognize, bind and phagocytose wear debris, while osteoclasts resorb mineralized bone matrix. Monocytes or macrophages are important precursory cells in the regulation of bone metabolism and destruction.

Multinucleation is an essential step in the differentiation of osteoclasts and for osteolysis as mononucleated macrophages may not resorb bone efficiently. Polykaryon formation from mononuclear precursor is an important step in the osteoclast differentiation. Osteoclasts are the bone resorbing cells, which play an important role in the regulation of both healthy and diseased bone. As fusion molecules, ADAMs may be involved in cell-cell fusion processes and in myoblast fusion and possibly also in osteoclast fusion (Huovila et al. 1996, Galliano et al. 2000, Huppertz et al. 2006). Similar cell fusion is also essential for the formation of multinuclear foreign body giant cells typical for aseptic loosening.

Bone is a dynamically remodeling tissue; the homeostasis is dependent on balanced bone resorption and formation. This soft tissue remodeling is adapted to structural and mechanical environment changes as a phenomenon of functional adaptation (Fung, 1990). Mature osteoclasts initiate the activation-resorption-formation (ARF) cycle and demineralization of bone and collagen degradation. Once activated, osteoclasts secrete protons and proteinases. Proteinases have been regarded as markers for inflammation and enhanced tissue remodelling. MMPs and cathepsin K are rate limiting and therefore, the most important proteinases in this process (Delaisse et al. 2000, Everts et al. 2006). The presence of various collagenases and cathepsin K in the synovial membrane-like interface membrane is necessary for collagen degradation.
5. REVIEW OF THE LITERATURE

5.1 THR and aseptic loosening

5.1.1 Total hip replacement

Today nearly one million total hip replacements (THR) are carried out worldwide each year (Konttinen et al. 1997). Primary osteoarthritis and joint destruction caused by rheumatoid arthritis are the most frequent causes of hip pain and invalidity (Maynard et al. 1995). Severe primary or secondary osteoarthritis can be effectively treated using THR, which has considerably improved the quality of life of these patients (Laupacis et al. 1993). In the 1960s John Charnley developed the low-friction arthroplasty design consisting of a small-diameter metallic femoral head articulating with a polymeric acetabular cup (Charnley 1961). A small head was selected to minimize the area of the gliding surface and thus the formation of wear debris of the polymeric polyethylene cup. This concept was the most successful surgical procedure developed for replacing the hip joint (Lehtimäki et al. 1997, Heisel et al. 2004). The vast majority of total hip replacement prosthesis consists of femoral and acetabular components. Femoral stem is fixed to femoral medullary canal and is made titanium or chromiumcobalt. The stem is usually modular i.e. it consists of a separate head (ball). Acetabular cup is fixed to pelvis and is made of polyethylene or metal (Figure 1). The cup can be either monoblock or modular (Figure 1). Monoblock cups are made of metal. Modular cups contain metallic shell and liner. Liner (inner part) is made of polyethylene, metal (chromiumcobalt) or ceramic. Femoral heads are usually made of either metal or ceramic. Thus, typical bearing surfaces are metal/ceramic-on-polyethylene, metal-on-metal and ceramic-on-ceramic. After surgery, fixation of uncemented components is based of friction. Later, bone ingrows on implants rough surface. Cemented implants are fixed using "bone cement" (polymethylmethacrylate) and achieve immediately during surgery their maximal stability. In younger patients, survival rate of uncemented implants has been better when aseptic loosening has been end-point (Mäkelä et al. 2008a, Mäkelä et al. 2008b). THR is a cost-effective artificial treatment with remarkable durability (Callaghan et al. 2004).

Figure 1. Conventional uncemented total hip prosthesis with metal-on-polyethylene bearings

5.1.2 Aseptic loosening
The most common long-term problem that restricts the patient’s daily activities after THR is pain associated with aseptic loosening and/or osteolysis. When a total hip prosthesis loses adequate fixation to bone it usually causes increasing pain and distinct changes develop into the radiographs. Extensive localized bone resorption resulting in loosening without infection was named aseptic loosening. Poor fixation leading to micromotion and the lack of initial stability and osseointegration of the implant are important factors of later symptomatic loosening (Munuera et al. 1992, Goodman et al. 1994). Mechanical cyclic loading, together with adverse host reaction to ultra-high molecular weight polyethylene wear debris and other foreign wear particles have been proposed as the main reasons for aseptic implant loosening. Implantation with the artificial joint leaves an open wound which is not replaced by scar tissue, because of the continuous movement of the prosthetic devices in the body (Konttinen et al. 1998). Wear debris produced from the THR cause a chronic inflammatory disorder, which can lead to peri-implant osteolysis. Aseptic loosening has been shown to be associated with this chronic foreign body type inflammation, which leads to activation of the local inflammatory cells, in particular those of the monocyte/macrophage lineage (Lassus et al. 1998, Shen et al. 2006). Synovial membrane-like interface tissue exists between the prosthesis/cement and host bone where inflammatory mediators are produced and contribute to periprosthetic osteolysis and implant loosening. It is evident that several inflammatory cells activated by foreign body reaction and their proinflammatory mediators like cytokines and degradative enzymes contribute to the pathogenesis of chronic aseptic inflammation and implant loosening. Macrophages and foreign body giant cells in the interface membrane surrounding loosened implants express cytokines associated with osteoclastic cells (Neale and Athanasou 1999). Some cytokines, e.g., interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α, have been detected in periprosthetic tissue in patients with loosening of the prosthesis (Konttinen et al. 1996, 1997).

5. 1. 3 Synovial membrane-like interface tissue

Synovial membrane-like interface tissue exists between the host bone and prosthesis. It is characterized by a cell-rich foreign body reaction although some areas are characterized by fibrosis containing mainly collagen fibers and elongated fibroblasts. Thus, there are two distinct morphologic areas a cell rich area containing macrophages and multinucleate giant cells, and fibrous area consisting of collagen fibres and elongated fibroblasts. A synovial lining-like structure is usually one to three cell layers thick and consists of fibroblast- and macrophage-like cells (Goldring et al. 1986, Jiranek et al. 1995, Goodman et al. 1998).

Micromovement of the implants may lead to the formation of first fibrous implant capsule and later synovial memebbrane-like interface tissue. Micromotion of the THR prosthesis might be due to necrosis produced in the superficial bone layer during implantation and cementing (Aspenberg et al. 1998), while implant components appear more stable in cementless prosthesis because of bone ingrowth (Little et al. 2006). Cyclic loading of a poorly fixed prosthesis may aggravate necrosis of peri-implant bone and prevent healing by bone fusion in cemented implants, whereas it can be healed in uncemented implants when motion stops. Early loading of prosthesis can also contribute to fibrous tissue ingrowth (Jasty et al. 1997). The pH in the interface tissue is low enough to lead to demineralization of peri-implant bone.

5. 2 Biomaterials and wear particles

Hip joint is loaded during our daily activities. Because artificial hip contains bearing surfaces, motion leads to friction and formation of wear debris and foreign bodies (Konttinen et al. 2005).
Such particles are particularly formed between the head and the cup, later is usually prepared of ultrahigh molecular weight polyethylene (UHMWPE) (Konttinen and Santavirta 2003). Foreign bodies are also formed elsewhere, e.g. between metal contact surfaces of stem vs. head, head vs. modular neck and polyethylene liner and acetabular shell. Debris, together with movement caused by loading, leads to irritation (Konttinen et al. 2005). Particles of prosthetic material stimulate host cells to release cytokines, which may cause chronic inflammation, bone loss and loosening of the prosthesis.

5.2.1 Biomaterials

Currently metals, polymers and ceramics are used as biomaterials of the different components of hip replacement implants (Katti 2004). The standard for hip arthroplasty is to use polymethylmethacrylate (PMMA) fixed UHMWPE acetabular cup and a femoral stem made of metal (Bauer et al. 1999). However, use of uncemented implants is increasing rapidly. Stainless steel proved to be too corrosive and has relatively low fatigue strength and elasticity. Metals currently used for THR are able to carry sufficient load and resist bending and fatigue. For hip replacement, Co-based alloys and TiAl6V4 are commonly used. Cobalt-based materials are nonmagnetic and relatively corrosion resistant and wear resistant. The favourable biocompatibility and high corrosion resistance of titanium make it a good choice for femoral stems (Head et al. 1995). However, titanium is not suitable for PMMA fixation due to its low resistance to abrasion, fretting and corrosion (Pohler 2000).

Polymers are classified as biostable, bioabsorbable and biodegradable polymers. Bone cement is polymerized methylmethacrylate, which is produced by mixing PMMA polymer powder and monomeric methylmethacrylate liquid in the presence of a catalyst. Under high tension or shear stress bone cement particles are formed. As a kind of conventional ceramics, alumina was approved as a material of ceramic-on-ceramic articulating hip for marketing by the United States Food and Drug Administration (FDA) in the United States in February of 2003, while ceramic-on-ceramic implants have been used in Europe approximately 25 years. Hydroxyapatite (Ca10(PO4)6(OH)2) has good biocompatibility and forms a tight bond to periprosthetic bone, but has poor mechanical properties and brittleness restricts its applications (Bagambisa et al. 1993).

The concept of biocompatibility refers to how harmonious is the implanted prosthesis in the body in host tissue and the capability to resist adverse tissue responses. The degree of biocompatibility of the implant material determines the type of tissue response. The less biocompatible the implant materials are, the more adverse host tissue responses they will produce. Using wear resistant and biocompatible materials is considered to increase longevity of the THR. The main direction of developing orthopedic implants or materials is to pursue higher biocompatibility. For example, there is an attempt to increase biocompatibility using hard-on-hard bearing surfaces in order to decrease amount of wear (Santavirta 2003).

5.2.2 Wear particles

Prosthetic wear particles derived from implants are foreign body materials. The foreign body response seen in tissue samples is often initiated by foreign materials not easily removed or degraded. Therefore, wear particles are the main reasons for aseptic loosening and failure of THR implants. The host response to wear particles leads to osteolysis and the extent of osteolysis usually increases as the rate of wear increases (Dumbleton et al. 2002). Ultramicron size polyethylene particles are particularly likely to cause “particle disease”. Macrophage is the first cell type which responds to wear particles. The amount of polyethylene debris in a microscopic
field was directly related to the number of macrophages that was visible by light microscopy (Schmalzried et al. 1992). When rubbing against metal femoral head, wear particles of UHMWPE are produced. The sizes of the UHMWPE particles isolated from tissues retrieved from failed total hip replacements has been in the range of 0.1–1000 µm diameter for patients, the maximum frequency distribution of the particles being in the range 0.1-0.5 µm, whereas analysis of the mass distribution revealed that the majority of the mass fell into size range >10 µm (Tipper et al. 2000, Howling et al. 2001). The mean size of the polyethylene particles was 0.5 µm, and of the metal particles 0.7 µm in metal-on-UHMWPE bearing surface. Wear particles from total hip prosthesis in the phagocytosable size range of 0.3–1.0 µm appear to be the most biologically active (Green et al. 1998, Ingham et al. 2005). Although larger fragments are relatively few, they contribute heavily to the total wear volume. A particle with size less than 7 µm in diameter is engulfed, whereas those greater than 7 µm are generally assumed to be too large to be phagocyted by macrophages and too large to contribute to the biological responses leading to osteolysis (Goodman et al. 1990). Particles of all biomaterials which are produced from bearing surface cause adverse biological reactions in periprosthetic tissues, which leads to the formation of osteolytic foreign body granulomas, inhibition of the bone formation and joint fluid production.

The environments around the articulating surfaces and body interfaces may contribute to the generation of wear particles (Maloney et al. 1995). Tribology deals with friction, lubrication, and wear around the counterface. The wear by adhesion, abrasion, and corrosion between the primary bearing surfaces is considered the most important source of wear particles (Schmalzried and Callaghan 1999). Cyclic loading and micromotion between interfaces are considerable factors contributing to wear (Aspenberg and Herbertsson 1996, Konttinen et al. 2005). More than three million steps per year potentially generate hundreds of billions of polyethylene and metal particles. Abrasive wear occurs when surfaces of different hardnesses move against each other, for example, the harder metal surface scratches the surface of the softer UHMWPE material. In total hip prostheses polyethylene wear is an important source of abrasive wear as the metallic femoral head is so much harder than the polyethylene. Polyethylene particles are transported by pseudosynovial fluid to the synovial membrane-like interface membrane where they are phagocytosed by macrophages. The macrophages then release chemokines, inflammatory cytokines and other mediators that stimulate the development of an inflamed granulomatous tissue adjacent to the bone. Eventually, osteoclasts are formed and/or activated to resorb bone leading to peri-implant osteolysis and loosening of the prosthesis (Shen et al. 2006). Many cytokines are present in the interface tissue around loosening implants and different types of prosthetic particles may induce different patterns of cytokines. Chemical composition of wear particles modulates the biological response, for example, wear particles of titanium alloys (TiAlV or TiAlNb) containing different concentrations of vanadium or niobium induced the expression of different osteolytic factors (Rogers et al. 1997). Particle surface chemistry also affects the activation of macrophages cytokine and enzyme production (Xing et al. 2002). Understanding the mechanism and effects of different biomaterials regulating osteoclast formation and activity may provide a way of selecting suitable biomaterials that should prolong the life time of implants.

5. 3 Osteoclastogenesis

Osteoclasts are bone resorbing cells, which play an important role in the regulation of both healthy and diseased bone. They are derived from haematopoietic cells of the mononuclear phagocyte progenitor (Burger et al. 1982). It has been earlier described that both macrophage-colony stimulating factor (M-CSF) (Xu et al. 1997) and receptor activator of nuclear factor kappa B ligand (RANKL) (Mandelin et al. 2003) found in the interface tissue are necessary to stimulate osteoclastogenesis (Fuller et al. 1998, Haynes et al. 2001). Tissue destructive factors such as
hydrochloric acid and proteinases (e.g. cathepsin K) lead to the dissolution of bone mineral (hydroxyapatite) and bone tissue.

5.3.1 Macrophage activation in aseptic loosening

Wear particle and cyclic load may together contribute to the activation of macrophages and an enhanced production of a set of cytokines, such as IL-1, IL-6, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), prostaglandin E₂ (PGE₂), M-CSF and transforming growth factor-α (TGF-α) (Ferrier et al. 2000, Green et al. 1998, Matthews et al. 2001). Use of hip implants always lead to the production of implant-derived particles, of which polymethylmethacrylate (PMMA) particles and the “cement disease” first draw attention and lead to the development of uncemented hip implants to avoid this complication. Later, when also other particles were found to be able to induce such reactions, the name of the condition was changed to “particle disease”. Thus, metal and especially ultra-high molecular weight polyethylene wear debris were also shown to be able to induce this host response. Wear debris leads to host reaction, to produce multinuclear giant cells from activated macrophage precursors as part of a foreign body reaction. Here the noxious stimulus, however, is of inorganic nature and can not be eliminated by the phagocytosing cells. Therefore, the response produced is chronic to its nature. Metallic implants are always surrounded by implant capsule, which in loosening develops into a synovial membrane-like interface membrane. It contains three principal cell types, macrophages, giant cells and fibroblasts, but naturally also blood and probably lymphatic vessels as well. Large amounts of wear debris are always detected extra- and/or intracellularly in interface tissue. Adverse responses of tissue to wear debris, fluid pressure waves caused by micromotion, proteinases, effective joint space and mechanical failure lead to the formation of synovial membrane-like interface tissue characterized by a foreign body reaction (Santavirta et al. 1991). Giant cells originate from the fusion of mononucleated precursors that belong to the mononuclear phagocyte lineage (Vignery 2000). As part of the host response, circulating monocytes make an initial tethering contact to activated endothelial cells and finally transmigrate to tissue in great numbers to form macrophages in order to get rid of particles. Macrophages activated by particles can secrete chemotactic cytokines so that they may play a role in induction of endothelial cell adhesion or the recruitment of osteoclast precursors from the vasculature by cytokine signaling (Frokjaer et al. 1995). The macrophage activation is influenced by particle characteristics, like their size, shape, rigidity, charge and chemical structure. Activated macrophages participate in the formation of osteoclasts. Soluble stimuli, like M-CSF and RANKL or pseudosynovial peri-implant fluid, can also induce macrophages to form osteoclasts (Mandelin et al. 2005).

5.3.2 Fusion of osteoclast progenitors

Particles, together with cyclic loading and implant-host interface micromovement are associated with macrophage accumulation and activation. Cytokines released by activated macrophages in turn stimulate monocyte recruitment and osteoclast precursor (monocyte/macrophage) proliferation and their differentiation in the interface tissue. The large numbers of macrophages present in the interface tissues have a potential capacity to form osteoclasts (Sabokbar et al. 1997). A key step in the giant cell formation is the fusion of mononuclear precursor cells together. Cell fusion need some preceeding preparatory steps, including cell accumulation, cell adhesion and membrane fusion. Monocyte trafficking and cellular movement regulated by chemokines and cytokines are essential for cell accumulation (Mackay et al. 2001, Muller et al. 2001). Both the β1 and β2 integrins play important role in monocyte/macrophage adhesion (McNally et al. 2002). Till now, membrane fusion is not well understood at the molecular level. The study of fusion proteins derives from studies of the viral fusion proteins, found in the membranes of enveloped viruses.
(Hernandez et al. 1996). Fusion molecules mediate membrane fusion in part through overcoming the repulsive forces of lipid bilayers. Macrophages are activated during multinuclear cell fusion and fusion-specific molecules may be induced in polykaryon-directed macrophage activation (Vignery et al. 2000, Ma et al. 2003).

5. 3. 3 Molecules involved in osteoclast formation

Activated macrophages produce high level of M-CSF which is important for osteoclast formation and peri-implant bone resorption. M-CSF is also essential for the monocyte–macrophage lineage cells proliferation, differentiation and survival (Flanagan et al. 1998). M-CSF was found in synovial-like membrane interface tissue and in the synovial fluid obtained from patients with aseptic loosening (Xu et al. 1997). It is important to note that M-CSF receptor is also present on the surface of macrophages and foreign body giant cells in the peri-implant tissues so the locally produced M-CSF can act in an auto- or paracrine fashion (Neale et al. 1998).

In osteobiology RANKL is an essential cytokine, which belongs to TNF superfamily. Haynes and coworkers demonstrated for the first time that arthroplasty-derived adherent cells from revision tissue samples including macrophages and fibroblasts expressed RANK (receptor activator of nuclear factor kappa B) and RANKL (Haynes et al. 2001). RANKL together with M-CSF induce human osteoclast formation in vitro, but it may be that osteoclasts are formed in a RANKL independent way supported by interleukin-6 and interleukin-11(Kudo et al. 2003). Particle-related inflammatory response in the periprosthetic tissues after total hip arthroplasty is associated with up-regulation of the RANKL/RANK/OPG (osteoprotegerin) system (Mandelin et al. 2003).

RANK belongs to the TNF receptor family and is RANKL receptor on osteoclast precursors (Anderson et al. 1997). RANK mRNA is present on osteoclasts and/or foreign body giant cells in interface tissues (Crotti et al. 2004). RANK is capable of initiating osteoclastogenic signal transduction after ligation with RANKL expressed by osteoblasts, fibroblasts, and bone marrow stromal cells or found in soluble form in the interstitial fluid. TNF-α via its type 1 receptor stimulates RANKL induced osteoclastogenesis by coupling of RANK and signal transduction (Zhang et al. 2001). Binding of RANKL to RANK activates several distinct cytoplasmic signalling pathways that lead to monocyte fusion and further activation. OPG, osteoclastogenesis inhibitory factor, is a secreted member of the TNF receptor family that lacks a transmembrane domain. OPG is expressed in human osteoblasts and stromal cells and it inhibits RANKL-related osteoclastogenesis (Simonet et al. 1997). OPG also inhibits osteoclast formation induced by joint fluid from patients with failed total hip arthroplasty (Itonaga et al. 2000). The balance of RANKL and OPG in peri-implant tissue may correlate with biocompatibility of implant and interface tissue (Mandelin et al. 2003).

Table 1. Cytokines and growth factors that influence osteoclast differentiation and activity

<table>
<thead>
<tr>
<th>Factors</th>
<th>Effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Receptor activator of nuclear factor kappa B ligand</td>
<td>induces osteoclast formation, differentiation and activation</td>
<td>Lacey et al. 1998, Haynes et al. 2001</td>
</tr>
<tr>
<td>Macrophage-colony stimulating factor</td>
<td>stimulates osteoclast differentiation, migrate to resorptive sites, maintain osteoclast survival</td>
<td>Neale et al. 2002, Sundquist et al. 1995, Fuller et al. 1993</td>
</tr>
<tr>
<td>Tumor necrosis factor-α and -β</td>
<td>stimulates the formation of osteoclast, bone resorption</td>
<td>Pfeilschifter et al. 1989</td>
</tr>
<tr>
<td>Enhancers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Interleukin-1</td>
<td>induces bone resorption and osteoclast-like cell formation</td>
<td>Pfeilschifter et al. 1989</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>induces osteoclast formation</td>
<td>Kurihara et al. 1990 Roodman et al. 1992</td>
</tr>
<tr>
<td>Interleukin-7</td>
<td>increases osteoclast formation, induces bone loss</td>
<td>Ross 2003 Toraldo et al. 2003</td>
</tr>
<tr>
<td>Interleukin-11</td>
<td>induces osteoclast formation</td>
<td>Girasole et al. 1994</td>
</tr>
<tr>
<td>Interleukin-17</td>
<td>stimulates osteoclast formation, increased bone resorption</td>
<td>Van bezooijen et al. 1999</td>
</tr>
<tr>
<td>Transforming growth factor-α</td>
<td>increases bone resorption</td>
<td>Yates, et al. 1992</td>
</tr>
<tr>
<td>Epidermal growth factor</td>
<td>stimulates bone resorption, enhances osteoclast recruitment</td>
<td>Saddi et al. 2008</td>
</tr>
<tr>
<td>Vascular endothelial growth factor</td>
<td>activates osteoclast migration, stimulate bone resorption</td>
<td>Henriksen et al. 2003 Zhang et al. 2008</td>
</tr>
<tr>
<td>Insulin-like growth factor-1</td>
<td>stimulates osteoclast differentiation</td>
<td>Wang et al. 2007</td>
</tr>
<tr>
<td>Platelet-derived growth factor</td>
<td>stimulates bone resorption</td>
<td>Zhang et al. 1998</td>
</tr>
<tr>
<td>Bone morphogenetic protein-2</td>
<td>induces osteoclast differentiation and survival</td>
<td>Itoh et al. 2001</td>
</tr>
<tr>
<td>Bone morphogenetic protein-7</td>
<td>induces osteoclastogenesis and recruitment of osteoclast</td>
<td>Hentunen et al. 1995</td>
</tr>
<tr>
<td>Leukaemia inhibitory factor</td>
<td>stimulates osteoclast differentiation</td>
<td>Heymann et al. 1997 Richards et al. 2000</td>
</tr>
<tr>
<td>Fibroblast growth factor-2</td>
<td>promotes osteoclast recruitment, formation, differentiation</td>
<td>Collin-Osdoby et al. 2002</td>
</tr>
<tr>
<td>Fibroblast growth factor-18</td>
<td>induces osteoclast formation, stimulate bone resorption</td>
<td>Shimoaka et al. 2002</td>
</tr>
<tr>
<td>Prostaglandin E₂</td>
<td>increase osteoclast acidity, induce osteoclast differentiation</td>
<td>Anderson et al. 1986 Liu et al. 2006</td>
</tr>
<tr>
<td>Monocyte chemotactic protein-1</td>
<td>induces osteoclast differentiation, chemotactic recruitment</td>
<td>Kim et al. 2006 Capellen et al. 2002 Li et al. 2007</td>
</tr>
<tr>
<td>Macrophage inflammatory protein-1</td>
<td>induces osteoclast differentiation, survival, activation</td>
<td>Oba et al. 2005 Okamatsu et al. 2004</td>
</tr>
<tr>
<td>Stromal derived factor-1</td>
<td>Promotes chemotactic recruitment, stimulates cell fusion</td>
<td>Liao et al. 2005 Wright et al. 2005 Yu et al. 2003</td>
</tr>
<tr>
<td>Osteoprotegerin</td>
<td>inhibits osteoclastogenesis inhibits osteoclastic bone resorption</td>
<td>Theoleyre et al. 2003 Hamdy et al. 2005</td>
</tr>
<tr>
<td>Interferon γ</td>
<td>suppresses the formation and maturation of osteoclasts</td>
<td>Fox et al. 2000 Takahashi et al. 1986</td>
</tr>
<tr>
<td>Interferon β</td>
<td>inhibits osteoclast differentiation</td>
<td>Alliston et al. 2002</td>
</tr>
<tr>
<td>Interleukin-4</td>
<td>inhibits osteoclasts formation inhibits bone resorption</td>
<td>Shioi et al. 1991 Riancho et al. 1993</td>
</tr>
<tr>
<td>Interleukin-10</td>
<td>inhibits osteoclastogenesis</td>
<td>Evans and Fox, et al. 2007</td>
</tr>
</tbody>
</table>
Interleukin-13 inhibits osteoclast differentiation and bone resorption [Palmqvist et al. 2006]
Interleukin-18 inhibits osteoclast formation [Horwood et al. 1998]
Nitric oxide inhibits bone resorption [Brandi et al. 1995]
Endothelin inhibits bone resorption [Alam et al. 1992]

<table>
<thead>
<tr>
<th>Factors</th>
<th>Effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25-dihydroxyvitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>induces osteoclast formation</td>
<td>Thavarajah et al. 1991</td>
</tr>
<tr>
<td>Parathyroid hormone and related peptide</td>
<td>osteoclast activation stimulatory effects</td>
<td>Blair et al. 2004</td>
</tr>
<tr>
<td>Glucocorticoids</td>
<td>stimulate osteoclast generation increase bone resorption</td>
<td>Sivagurunathan et al. 2005, Yao et al. 2008</td>
</tr>
</tbody>
</table>

TABLE 2. Dual factors

<table>
<thead>
<tr>
<th>Factors</th>
<th>Effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transforming growth factor β</td>
<td>Low concentrations stimulates osteoclast formation, high concentrations inhibitory</td>
<td>Shinar et al. 1990, Hattersley et al. 1991, Janssens et al. 2005</td>
</tr>
</tbody>
</table>

Interleukin-11 is an important component of cytokine network mediating osteoblast-osteoclast communication in periprosthetic osteolysis and in the loosening of THR implants [Xu et al. 1998]. In the presence of interleukin 11 osteoclasts showed a high degree of resorption [Girasole et al. 1994] (Table 1, 2, Figure 2).
5. 4 ADAMs and cell fusion

5. 4. 1 ADAMs

The ADAMs (an abbreviation for **A** Disintegrin **A**nd **M**etalloproteinase) are a family of multidomain transmembrane glycoproteins which always have a disintegrin and a metalloprotease domain. At present, more than 30 members of ADAM family (Table 2) and 19 different ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) have been found (Blobel *et al.* 2005, Jones *et al.* 2005). ADAMTS also have a thrombospondin TS domain. Based on studies it is clear that ADAMs mRNAs are present in a wide range of mammalian tissues. The extracellular structure of ADAMs shares a high sequence homology and domain organization with snake venom metalloprotease (Schlondorff *et al.* 1999). The term disintegrin was first used to describe viper venom protein which binds tightly to the platelet integrin receptor and blocks the interaction of integrin with its ligand to inhibit platelet aggregation (Gould *et al.* 1990, McLane *et al.* 1998). They also seem to be involved in some diseases such as inflammation and cancer. ADAMs belong to the zinc protease superfamily. Their metalloprotease domains are proteolytically active and can degrade extracellular matrix components and can shed other proteinases, adhesion molecules, cytokines and their receptors (Black *et al.* 1998). The disintegrin domain of ADAMs is a ligand for integrins. Some ADAMs contain a putative fusion peptide in the cysteine-rich domain that is involved in both membrane fusion and osteoclast formation (Figure 3) (Abe *et al.* 1999, Seals *et al.* 2003). Viral fusion peptides are short segments of mostly hydrophobic amino acids found in a transmembrane subunit of viral fusion proteins which are primarily involved in virus-host target cell fusion. A fusion peptide penetrates host cells like a sword, penetrating the lipid bilayer of the host cell. Thus, the anchoring fusion peptide propels the virion close enough to the host target cell membrane to be able to trigger virus-host cell fusion.
(White, 1992, 1995, White et al., 1996). ADAM1, 5, 8, 9, 11, 12, 14 and 20 have been reported to have this hydrophobic homologous sequence (Huovila et al. 1996, Duffy et al. 2003).

Figure 3. Domain structure of ADAMs (Modified from Seals et al. 2003).

Table 3. Human ADAMs

<table>
<thead>
<tr>
<th>ADAM</th>
<th>Common name(s)</th>
<th>Potential functions</th>
<th>Expression</th>
<th>MP active</th>
<th>Integrin binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>fertilin-β, PH-30 β</td>
<td>sperm/egg binding/fusion</td>
<td>testis²</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>epididymal apical protein 1</td>
<td></td>
<td>epididymis²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>murine cell-surface antigen 2, CD156</td>
<td></td>
<td>granulocytes/ monocytes³</td>
<td>✓ (d)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>meltrin-γ, metalloprotease/disintegrin/cysteine-rich protein 9</td>
<td>sheddase, cell migration/fusion</td>
<td>widely expressed⁵</td>
<td>✓ (d)</td>
<td>✓ (d)</td>
</tr>
<tr>
<td>10</td>
<td>kuzbanian, mammalian disintegrin-metalloprotease, SUP-17</td>
<td>sheddase; cell fate determination</td>
<td>widely expressed⁶</td>
<td>✓ (d)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>metalloprotease/disintegrin/cysteine-rich protein</td>
<td>putative tumor repressor</td>
<td>brain³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>meltrin-α</td>
<td>sheddase, myoblast fusion</td>
<td>widely expressed⁸-10</td>
<td>✓ (d)</td>
<td>✓</td>
</tr>
<tr>
<td>15</td>
<td>metargidin, metalloprotease/disintegrin/cysteine-rich protein 15</td>
<td>cell/cell binding</td>
<td>widely expressed³¹</td>
<td>✓ (p)</td>
<td>✓</td>
</tr>
<tr>
<td>17</td>
<td>Tumour necrosis factor-α convertase</td>
<td>sheddase</td>
<td>widely expressed¹²</td>
<td>✓ (d)</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>testis metalloprotease/disintegrin/cysteine-rich protein III</td>
<td></td>
<td>testis¹³</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>meltrin-β, metalloprotease and disintegrin dendritic antigen marker</td>
<td>sheddase, dendritic cell development</td>
<td>widely expressed³¹,⁴⁴</td>
<td>✓ (d)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>testis</td>
<td></td>
<td>testis¹⁵</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>testis</td>
<td></td>
<td>testis¹⁵</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>metalloprotease/disintegrin/cysteine-rich protein 2</td>
<td></td>
<td>brain²⁰</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>metalloprotease/disintegrin/cysteine-rich protein 3</td>
<td></td>
<td>brain²⁷</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>metalloprotease/disintegrin/cysteine-rich protein-L</td>
<td>immune surveillance</td>
<td>epididymis, lung lymphocytes¹⁸-²⁰</td>
<td>✓ (d)</td>
<td>✓</td>
</tr>
<tr>
<td>29</td>
<td>testis</td>
<td></td>
<td>testis¹²-²²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>testis</td>
<td></td>
<td>testis¹⁷</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>33</td>
<td>genetically linked to asthma</td>
<td>widely expressed²³</td>
<td>✓ (p)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
ADAM12 is a member of ADAMs family, which has been implicated in cell adhesion, cell fusion and signaling (Wolfsberg et al. 1995). ADAM12 comprises a pro-domain, a metalloprotease domain, a disintegrin domain, a cystein-rich domain, an epidermal growth factor (EGF)-like domain, and a transmembrane domain with an attached cytoplasmic tail. The disintegrin domain contains a non-RGD (arginine-glycine-aspartic acid) group, which has been shown to support cell adhesion mediated through integrin receptors (Zolkiewska et al. 1995). Cysteine-rich domain can act as a ligand for cell-adhesion molecules which can promote the fusion protein activity of this domain (Schlondorff et al. 1999). The cytoplasmic domain contains several motifs potentially involved in fusion associated signal transduction (Huovila et al. 1996). Furthermore the cytoplasmic tail interacts with the actin cytoskeleton by directly binding to α-actinin-1 and -2 (Galliano et al. 2000). α-actinins form direct links between the cellular actin cytoskeleton and cytoplasmic tail of ADAM12. This may explain some of the gross changes in cell morphology that occur during the cell fusion. Thus, the disintegrin, cysteine-rich and/or EGF repeat regions and the cytoplasmic tail of ADAM12 seem to promote cell fusion. It was reported that myotube fusion requires fusion molecule ADAM12 (meltrin-α, Yagami-Hiromasa et al. 1998), while macrophage-derived giant cells and osteoclasts is the result of cell-cell fusion of mononuclear precursors via ADAM8 (Choi et al. 2001).

Another ADAM, ADAM9 has been found to be involved with proteolysis, cell adhesion and cell fusion. The metalloprotease domain of ADAM9 contains structures necessary for metalloprotease activity. Cysteine-rich domain can be involved in macrophage membrane fusion (Puissegur et al. 2007). All the cysteine residues in the disintegrin and cysteine-rich domains are engaged in disulfide bonds and the overall disulfide bond configuration was found to be essential for the cell adhesive potential of the cell (White et al. 2003). It was shown that expression of ADAM9 mRNA enhanced by either RANKL+M-CSF or anti-CD98 antibody stimulation was associated with an accelerated monocytes fusion process (Namba et al. 2001). ADAM9, known as meltrin-γ, has a complex and multifunctional domain structure, may play multiple roles during the cell fusion procedure. Like ADAM1 (fertilin-α), it contains a fusogenic peptide in its cysteine-rich domain. Synthetic peptides corresponding to this fusion peptide, when reconstituted into liposome or incorporated in lipid bilayers, supported the proposed function in membrane fusion (Martin et al. 1998). The MMPs could be transported by cyclic loading and pseudosynovial fluid to the interface membrane from the site produced. There they can participate in the degradation of the extracellular matrix (ECM) and expansion of the effective joint space as an essential part of the aseptic loosening. ADAM9 has metalloproteinase domain with somewhat similar proteolytic functions as MMPs. The metalloprotease domain of ADAM9 contains cysteine switch, furin cleavage, catalytic and Met-turn sites (Birkedal-Hansen et al. 1995, Pei et al. 1995, Mohan et al. 2002). The catalytic site of metalloproteinases contains a zinc binding consensus sequence HEXXXH (Wolfsberg et al. 1993). It may cleave basement membrane components and degrade extracellular matrix in loosening hip implants. Cytokines and their receptors play important roles in aseptic loosening (Konttinen et al. 1997). ADAM9 may mediate shedding of both cytokines.
and their receptors and thus regulate their activity (Amour et al. 2002). Adhesive ECM molecules may bind to the surface of the implant and thus facilitate binding of the host cells to the surface of the implant. This may help binding of bone cells to the surface of the implant, which may facilitate osseointegration of the prosthesis. Degradation of ECM may impair this fixation to bone, extend the joint space and loosen the implant. The disintegrin domain of ADAM9 has been shown to interact with the \( \alpha_v \beta_5 \) integrin receptor (Zhou et al. 2001). Integrins regulate many cellular events, including adhesion and migration. The interaction between ADAM9 and \( \alpha_v \beta_5 \) has been shown to regulate IL-6 production (Karadag et al. 2006), while IL-6 produced in periprosthetic interface tissue may contribute to osteolysis of the nearby bone (Konttinen et al. 2002). The cytoplasmic tail of ADAM9 contains Src homology 3 (SH3) binding motifs (Weskamp et al. 1996) and may interact with actin cytoskeleton and participate cell signalling because SH3 associating with the actin cytoskeleton indicates that this domain might serve to bring together signal transduction proteins and their targets or regulators to the membrane cytoskeleton (Musacchio et al. 1992).  

5. 4. 2 Cell fusion

Membrane fusion belongs to the most fundamental processes in living organism. Membrane fusion events are generally classified in three types: intracellular fusion, virus–cell fusion and cell–cell fusion. Cell–cell fusion is required for processes as diverse as fertilization, the formation of bone and placenta, and myogenesis (Jahn et al. 2003). Formation of multinucleated cells has two main effects on macrophages: it increases the size of the cells and enhancing the number of nuclei. Multinucleation of macrophages accompanied with functional phenotype changes is an essential step in osteoclast formation because mononucleated macrophages do not resorb bone efficiently. From an energetic point of view, the advantage of multiple nuclei is that to transport proteins and/or RNA from the nucleus to where they are needed in the cell could save more energy by having more nuclei spread around the cell. This might be particular useful for foreign body giant cells in interface tissue to destroy wear particles or for osteoclasts to resorb bone. The higher number of nuclei the multinucleated giant cells have, the bigger is the size of the targeted particles or bone surface area, which can be phagocytosed and eventually destroyed (Vignery et al. 2005).  

In paramyxovirus-to-host cell fusion the fusing virion membrane and host cell membrane are first brought into close contact and docked to each other. This occurs with the help of the hemagglutinin-neuraminidase on the surface of the virus, which binds to its sialic acid-containing receptor on the surface of the host cell. This interaction triggers the close-by latent fusion protein (F protein) trimers to undergo conformational changes so that they expose their second hidden hydrophobic fusion peptide domain. These now exposed hydrophobic viral fusion peptides insert into the host (target) cell membrane. Two of the anchoring fusion proteins then again fold, now so that their two trimeric hydrophobic domains (the transmembrane domain in the virion membrane and the fusion peptide domain in the host cell membrane) aline in an anti-parallel fashion in a structurally strong 6-helix bundle. This power stroke brings the virion membrane and host cell membrane together leading first to exoplasmic virus-host cell fusion and formation and expansion of the initial pore between the virus and host cell (Lamb et al. 2006). Thus, the viral fusion protein helps the viral envelope to fuse directly with the plasma membrane of its host target cell (Moscona 2005).  

There are plenty of studies about virus–cell fusion. However, the number of studies of virally induced host cell fusion and syncytium formation between infected and non-infected cells are sparse. Fusion of virally infected cells with adjacent uninfected cells forms large multi-nucleated
cells called syncytia. The fused mass of the cells is called "syncytium". This virus induced host cell fusion is much more complex than virus–cell fusion, because there are many more receptors and glycoproteins on the cell surface than there are on the surface of an enveloped virus. Signal transduction and subsequent cell activation is an intricate multi-step mechanism mediating membrane fusion in which membrane lipids (phospholipids and cholesterol) are tightly involved. Interaction of host cells with neighboring cells bearing surface fusion molecules is required for syncytium formation. Syncytium formation and generalized cell fusion are potentially important mechanisms mediating virally induced cytotoxic effects because the giant cells die soon after they are formed (Lifson et al. 1986 a, b). Therefore, cell fusion of virally infected cells with neighboring cells to form multinucleated syncytia contributes to the pathogenic effects of virus. Multinucleated giant cells originating from monocyte-macrophages are associated with granulomatous lesions formed in response to viruses, bacteria or foreign bodies. We hypothesized, that such molecules of mammalian origin could contribute to virally induced host cell-host cell fusion and would also otherwise have already been recognized for their role in other, non-virally induced mammalian cell-cell fusion events.

Myoblast fusion is a prerequisite for skeletal muscle differentiation, characterized by mononucleated myoblasts fusing to multinucleated muscle fibers (Wakelam et al. 1985). Proteins involved in myoblast fusion include cell-adhesion molecules, metalloproteinases, calmodulin, protein kinases and phospholipases (Horsley et al. 2004). Meltrin-α (ADAM12) was cloned from a myoblast source. It showed a restricted expression pattern that correlated well with early skeletal muscle development and muscle regeneration (Borneman et al. 2000). Another important step of myoblast fusion is that the fusion competent cells rearrange their actin cytoskeleton.

In the process of macrophage fusion to osteoclast, RANKL instructs macrophage precursors to fuse. Other molecules identified as critical for the fusion include macrophage fusion receptor and its ligand CD47. They contribute to polykaryon formation as adhesion factors (Han et al. 2000, Helming and Gordon 2007). Dendritic cell specific transmembrane protein is also essential for multinucleation, but it need to trigger some membrane bound fusion molecules to initiate fusion (Yang et al. 2008, Vignery 2005). ADAMs might be just such kind of molecules which contain putative fusion peptide to start the fusion process.

5. 4. 3 Role of ADAMs in cell fusion

Cell–cell fusion reactions are critical in some developmental processes. Some ADAMs are expressed and participate in several cell fusion events, such as fusions of sperm–egg to form the zygote requiring ADAM1 and ADAM2 (Blobel et al. 1992, Cho et al. 1998), myoblasts to form myotubes regulating by ADAM12 (Galliano et al. 2000) and monocytes to form osteoclasts associating with ADAM8 (Mandelin et al. 2000), ADAM9 and ADAM12 (Verrier et al. 2004). In certain specialized tissues, such as placenta, fusion of cytotrophoblasts generates a protective and nutritive layer for the developing embryo (Huppertz et al. 2006). Proteins involved in sperm-egg fusion that may be considered as the most important cell fusion of life are ADAM1 and 2 (Waters et al. 1997). ADAM8 increases osteoclast formation when present in the fusion stage and it plays a role in the later stages of osteoclast differentiation (Choi et al. 2001). Meltrin-α (ADAM12) not only participates and regulates myoblast fusion but also osteoclast differentiation (Abe et al. 1999). Among all these ADAMs 8, 9, 10, 12, 15, 17, and 28, which are expressed in osteoclast precursors and in mature osteoclasts, ADAM8 and ADAM12 may play an important role in the early stages of osteoclast formation (Verrier et al. 2004).

Due to their role in other cell-cell fusion events, ADAMs form also good candidate molecules
which might participate in virus induced cell-cell fusion. Fusion competent viruses might overtake host cell fusion system temporarily to their own use by overexpressing host’s own fusion peptides to promote cell-cell fusion and spreading of virus by just infected one cell. Using this kind Trojan horse strategy virus can spread efficiently and safely.

5. 5 Bone destruction

Aseptic loosened implants are always surrounded by a synovial membrane-like interface membrane, which is characterized by chronic inflammation (Atkins et al. 1997). Synovial-like lining and the potential space, an extension of the joint space, is the factual sign of loosening. It was first demonstrated by Harris and coworkers who performed arthrographies of totally replaced hip joints just prior to revision operation. It was possible to trace spreading of the intra-articularly injected contrast medium to this extended joint space which in some cases seemed to surround the whole implant. This interface is rich in tissue destructive collagenolytic enzymes which play important role in peri-implant bone destruction. Localized bone loss and local bone erosions are mainly produced by osteoclasts (Gravallese et al. 1998). Increased osteoclastic bone resorption plays an important pathogenic role in aseptic loosening.

5. 5. 1 Osteoclast function

Bone is a dynamically remodeling tissue, whose homeostasis balanced by bone resorption and formation, regulated to maintain of bone quality (Blair, 1998). Bone resorption and formation are linked closely within a temporary anatomic structures called the basic multicellular unit (BMU) which consists of a cutting cone formed by a group of osteoclasts, followed by a group of osteoblasts with form mineralizing connective tissue, i.e. bone in the closing cone (Figure 4, Parfitt 2002, Hernandez et al. 1999). The BMU exists and moves, excavating and refilling across the surface of trabecular bone as an instruments of bone remodeling. Osteoblasts regulate locally the differentiation and function of osteoclasts in normal bone. Bone remodelling comprises osteoclast-mediated bone resorption and osteoblast-mediated bone formation, which are regulated by hormones, growth factors and locally by cytokines (Raisz, 1999). Cytokines regulate their target cells via cell surface-bound cytokine receptors utilizing different intracellular signal transducing pathways (Klinger et al. 1998). Bone resorption occurs within the resorption site, Howship’s lacuna, a tightly sealed zone between the ruffled border of the osteoclast and bone surface. Subsequently osteoclasts produce an acidic environment and collagenolytic acidic endoproteinases, in particular cathepsin K.

Figure 4. BMU travels through the bone tissue. Wi, represents width of the BMU; R represents rate of progression, expressed in units of BMUs per square millimeter of tissue per day; FP represents formation period (days); EP represents erosion period (days). The tail represents the new formed osteon (modified from Hernandez et al. 1999)
Cellular activity at the remodeling site proceeds in an activation-resorption-formation (ARF) cycles (Figure 5). Mature osteoclasts need to undergo activation to initiate this cycle (Väänänen et al. 2000). In response to bone deformation, fatigue-related microfracture or wear particles, a group of preosteoclasts are activated. These mononuclear cells attach to the bone via $\alpha_v\beta_3$ integrins and fuse to form multinucleated osteoclasts via fusion molecules.

The functional events relevant for osteolysis consist of osteoclast migration towards bone, followed by adherence to the bone surface, initiation of the resorptive process, dissolution of hydroxyapatite, degradation of organic collagen-rich matrix, transcellular removal of degradation products from the resorption lacuna, and finally return to the non-resorption stage. Osteoclast migration requires the formation of podosomes which are unique actin-based adhesion structures (Destaing et al. 2003). Peripheral podosomes fuse to form the actin ring that circles the osteoclast domain, where active bone resorption occurs (Lakkakorpi et al. 1993). $\alpha_v\beta_3$ integrin is essential to podosome rearrangement, actin ring formation and osteoclast polarization (Faccio et al. 2002). The activated osteoclast forms a tight junction and a ruffled border facing the bone surface (Teitelbaum 2000). These processes result in the formation of resorption pits (Everts et al. 2002). In this depression called Howship’s lacuna, proton pump function drops pH so that inorganic hydroxyapatite mineral matrix is dissolved and that the pH becomes optimal for cathepsin K-mediated collagen degradation (Everts et al. 1992, Farina et al. 2002).

Osteoclasts secrete both $\text{H}^+$-ions and cathepsin K important for osteoclastic bone resorption. Blair and coworkers (1989) and Väänänen and coworkers (1990) were the first who showed that it was vacuolar-type proton ATPase in osteoclasts that was responsible for mineral dissolution. Protons were pumped into the resorption lacuna using vacuolar $\text{H}^+$-adenosine triphosphatase (ATPase) localized in the ruffled border of osteoclasts so that osteoclasts are capable of generating acidosis to cause mineral dissolution necessary for bone resorption (Tuukkanen and Väänänen 1986, Väänänen et al. 1990). Cathepsin K, which has high proteolytic activity and localizes primarily in osteoclasts, is one of the few extracellular proteolytic enzymes capable of degrading collagen. Under low pH, cathepsin K exerts autocatalytic activation and collagenolytic activity (McQueney et al. 1997, Konttinen et al. 2001). The secreted form of cathepsin K may be a proenzyme, but in the extracellular environment outside the osteoclasts it can be autocatalytically activated (Claveau et al. 2000). Overexpression of cathepsin K has been reported to increase bone destruction, while in cathepsin K deficient mice, the bone resorption was impaired (Kiviranta et al. 2001, 2005). Therefore, in bone destruction process osteoclasts dissolve mineral by acid environment and degrade uncalcified organic matrix by cathepsin K or MMPs (Syggelos et al. 2001, Garnero et al. 1998). All the bone resorption byproducts are removed via transcytosis from the ruffled border facing to the bone to the other side of osteoclast to the extracellular space. (Salo et al. 1997, Nesbitt and Horton 1997, Mulari et al. 2003). Gap junction may also regulate osteoclast function by
regulating bone resorption, osteoclast survival and apoptosis (Ilvesaro and Tuukkanen 2003). Fibroblasts grow on bone surface during peri-implant osteolysis where they may cause bone dissolution by a similar mechanism by secreting H$^+$ ions, but not forming resorptive compartments like osteoclasts do. Therefore, fibroblasts in the local environment of peri-implant can cause degradation of the bone surface by lowering the pH and releasing enzymes, but the amount of resorption is minor so the contribution of the process to the peri-implant bone lysis may be small (Pap et al. 2003).

5. 5. 2 Collagen degradation

Collagens are major structural proteins in the extracellular matrix, with more than 20 different types. Tissues contain 3 types of collagens: 1) soluble (monomers); 2) collagenase-cleaved collagen in fibers; can be solublized with chymotrypsin; 3) intact collagen in fibers, this is the insoluble matrix. Bone is connective tissue, which contains type I collagen fibers embedded in an inorganic hydroxyapatite [Ca$_{10}$(PO$_4$)$_6$(OH)$_2$] matrix. Type I collagen is among the most abundant proteins in the body. It accounts for up to 90% of the dry weight of bone and it is also the major matrix protein of tendons, ligaments, and soft connective tissues. All collagens have common conformation, the mature alpha chain and a triple helical collagen monomer contains 1000 of amino acids. Glycine has to be located at every third residue of the triplet amino acid sequence to allow helix formation. This is enabled as the side chain glycine is the simplest and smallest. These Gly-X-Y amino acid triplets, in which the “X” position is frequently occupied by proline and the “Y” position by hydroxyproline residues, form a single α-chain, which is the smallest structural unit of collagen. Three α-chains form a triple helical collagen monomer. These collagen monomers align into a nearly three-quarter-overlapping collagen fiber, which is stabilized through intermolecular cross-links. These fibers form the collagen bundles and networks in tissues (Persikov and Brodsky, 2002). Collagen fiber matrix is winding into natural bone fixed by HAP (Ca$_{10}$(PO$_4$)$_6$(OH)$_2$). The elastic modulus of bone is intermediate between hydroxyapatite and collagen (Fratzl et al. 1998).

Two types of collagen degradation pathways have been discovered. One is the intracellular route mainly active in a physiological steady state and the other is extracellular route playing a major role in collagen matrix remodelling and various pathological conditions (Trackman et al. 2005). Collagenases play an important role in physiologic tissue remodeling and pathologic conditions. Imbalance of proteinases and their endogenous inhibitors may cause pathological collagen degradation. Once activated, osteoclasts secrete protons and proteinases at their attachment site, resulting in dissolution of bone mineral and degradation of the exposed collagen matrix in an acidic pH. Cathepsin K is probably the most important proteinases in this process (Konttinen et al. 2001). The presence of various collagenases and cathepsin K in the synovial membrane-like interface membrane is necessary for collagen degradation. Collagen destruction in interface membrane might be mechanically induced by the micromovement subjected to cyclic loading or walking, in part due to unisoelasticity of the implant and host bone (Konttinen et al. 2005). High mechanical loading leads to an increased collagenase-1 (MMP-1) production (Holliday et al. 1997). MMP-1 can only cleave collagen triple helix at the initial specific cleavage site between Gly$^{775}$-Leu/Ile$^{776}$ of the triple helix and acts as the rate-limiting enzyme in collagen proteolysis (Pardo et al. 2005). MMP-13 effectively degrades type II collagen and cleave type I collagen at an additional aminotelopeptide locus (Krane et al. 1996). It has been shown that MMP-1 and -13 increased in aseptic loosening (Takei et al. 2000, Syggelos et al. 2001). Macrophages produce most MMPs in prosthetic interface tissue (Hembry et al. 1995), though fibroblasts also participate (Takagi et al. 1998). Under low pH cathepsin K is able to catalyze collagenolysis (Garnero et al. 1998). Cathepsin K has both an intracellular lysosomal function and an extracellular function in
the degradation of collagen (Kafienah et al. 1998). It is preferentially and highly expressed in osteoclasts and its level increases upon osteoclast activation (Corisdeo et al. 2001). Cathepsin K is able to hydrolyze type I collagen and to cleave across the collagen triple helix (Drake et al. 1996). All these enzymes can potentially participate in pathological peri-prosthetic bone destruction and act in concert with each other.
6. AIMS OF THE STUDY

Our hypotheses are:

1) ADAM9 and ADAM12 participate in the formation of foreign body giant cells and osteoclasts in aseptic loosening. The formation of foreign body giant cells and osteoclasts occurs in the interface tissue, which is always formed between the implant and host bone in the association to aseptic loosening. Therefore, their expression should be increased in aseptic loosening compared to control synovial membrane.

2) We have earlier shown that peri-implant interface membrane contains several proteinases. We therefore thought that some of them could participate in the pathological collagenous tissue degradation in peri-implant tissues leading to an expanded joint space and implant loosening.

Specific aims of the research:

1) To study the regulation of selected markers and active substances in interface membrane around loosening hip implant together with in vitro studies using M-CSF and RANKL to stimulate cells in monocyte/macrophage lineage for cell-cell fusion. Molecules which were analysed are tentative fusion molecules (members in the ADAM family, namely ADAM8, ADAM9 and ADAM12).

2) To use a new method to assess the degree of collagen degradation and to determine if collagen indeed is degraded around loosening hip implants and which proteinases are associated with this degradation.

3) To develop a new parainfluenza virus type 2-driven model to study the expression and involvement of ADAM8, ADAM9 and ADAM12 in multinuclear giant cell fusion.
7. MATERIALS AND METHODS

7.1 Tissue samples

Synovial membrane-like interface tissue was collected from areas adjacent to osteolytic lesions from 7 females and 3 males undergoing revision THR due to aseptic loosening of prostheses. These ten samples were collected from the proximal bone-cement or bone-stem interfaces around femoral stems during revision THR operations (Fig 6. Grüen Zones 1 and 7; Grüen et al. 1979, DeLee et al. 1976). All patients suffered from aseptic loosening reported having hip/thigh pain and radiographs disclosed liner periprosthetic osteolysis. There were no major iatrogenic technical problems such as femoral malalignment or poor cement mantle present in our patients, which could have explained loosening. The patients did not have any clinical signs or laboratory findings, which would have indicated deep joint infection. The type of implant material used in all patients was cobalt alloy-based prosthesis. Nine patients were fixed with cement, while only one was uncemented. None of the patients had severe metallosis. Their mean age was 71 years (range 57-80 yr). Seven operations had been performed due to osteoarthritis, two for a sequeale of hip fracture and one secondary osteoarthritis caused by rheumatoid arthritis. Cefuroxime 1.5 gr was given intravenously 30-45 minutes before skin incision as a prophylaxis. In all revisions, three intraoperative cultures for aerobic organisms of joint fluid and interface tissues were obtained and they were negative. The mean time from primary to revision THR was 7.7 years (range 5-16 yr). Ten samples were obtained for comparison from patients undergoing primary THR performed for osteoarthritis. Seven of these patients were women and three were men. Their mean age was 62.5 years (range 47–67 yr). Another set of nine control synovial membrane samples were

Figure 6. X-ray picture of a loose total hip prosthesis and surgical zones around the implant.
obtained from hip fracture patients. Of these patients six were women and three men, with the mean age 67.4 years (range 39-89 yr). All samples were immediately immersed in physiological saline. Samples were snap frozen, embedded in Tissue-Tek OCT compound and stored at –70°C. Half of the sample was processed to paraffin after fixation in 4% formalin for 24 hours at room temperature followed by dehydration at room temperature, clearing and embedding in paraffin at 55°C. All samples were analyzed using immunohistochemistry, five representative samples from revision and primary THR operations were also used for in situ hybridization, in addition tissues from three patients in each category were extracted and analysed using western blotting.

7. 2 Cell culture

7. 2. 1 Osteoclast formation assay

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of three healthy blood donors of the Finnish Red Cross using Ficoll–Paque PLUS (Pharmacia Biotech, Uppsala, Sweden) density gradient isolation at 2500 g for 20 minutes at room temperature. Washed cells (1 × 10^7/well) were incubated in six-well plates with or without coverslips in 2 ml of minimal essential medium (MEM, HaartBio Ltd. Helsinki, Finland) Alpha Medium with Glutamax-1 (Invitrogen, San Diego, USA) supplemented with 10% fetal calf serum, 100 IU/ml of penicillin (Invitrogen) and 100 µg/ml of streptomycin (Invitrogen). After one hour, the non-adherent lymphocytes were washed away; the adherent monocytes were used for experiments and and they were stimulated with 25 ng/ml of rhM-CSF (R&D, Minneapolis, USA) and 40 ng/ml of rhRANKL (Alexis, Carlsbad, CA, USA, Paloneva et al. 2003) for 1, 3 7 and 14 days before they were analysed using immunofluorescence staining and flow cytometry. The media was replaced twice a week.

7. 2. 2 Viral infection of GMK, HSG and HSY cells

GMK, a kidney-derived epithelial-like cell line, is susceptible to PIV2 and was maintained in virological laboratories to generate PIV2 virions. It was obtained from the Helsinki University Central Hospital (HUCH) laboratory and maintained in minimal essential medium (MEM, HaartBio Ltd. Helsinki, Finland) containing 10 % (v/v) heat-inactivated foetal bovine serum and 2.2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (HaartBio) in 75 cm² culture flasks at 37°C and 5 % in the CO₂-incubator (Ammendolia et al., 2007).

HSG cell line derived from human submandibular gland (Shirasuna et al., 1981) and HSY cell line derived from human parotid gland (Yanagawa et al., 1986) were cultured at 37°C, 5 % CO₂-in-air in Dulbecco’s modified Eagle’s medium with nutrient mixture F-12 Ham (DMEM/F-12, Sigma, St. Louis, USA) supplemented with 10 % (v/v) fetal calf serum, 2.2 mM glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

PIV2 was obtained from the Helsinki University Central Hospital (HUCH) laboratory where it was used as a reference virus. The virus was grown in the GMK cells according to standard procedures (Richman et al., 1997). Medium was removed when the adherent GMK, HSG or HSY target cell cultures covered 70–90 % of the surface available for them in 75 cm² cell culture flasks. Cultures were washed with 1 ml of culture medium twice and then exposed to 1.5 µg/ml trypsin solution at +37°C and in 5% CO₂ for 5 minutes. The detached GMK, HSG or HSY cells were divided into 6-well plates, 1.2x10^6 cells per well. 23 µl PIV2 primary viral suspension, containing 2.7x10^7 infective units/ml per millilitre, was added to each 2.5 ml in cell culture well. The cells were harvested at 0 (before PIV2 was added), 2, 24 and 72 hours. In parallel, cells
cultured without PIV2 were harvested as controls at same time points as above.

7. 3 Immunohistochemistry

7. 3. 1 Immunohistochemical avidin-biotin-peroxidase complex staining

Ten consecutive 6 μm-thick cryostat sections were cut from each sample. The first and last sections were stained with hematoxylin-eosin to check the morphology, whereas the rest of the sections were used for immunohistochemistry. Sections were mounted on Dako TechMate capillary microscope slides (TechMate™, Dako, Glostrup, Denmark) and fixed in 4°C acetone for 15 minutes. Slides were washed in Dako ChemMate washing buffer and installed in a Dako TechMate Horizon immunostainer. Paraffin tissue sections were deparaffinized in xylene, rehydrated through a graded ethanol series and washed in 10 mM phosphate-buffered saline, pH 7.4 (PBS). Antigens were retrieved for 30 minutes using MicroMED T/T Mega Laboratory Microwave Systems (Milestone, Sorisole, Italy) in 0.01 M sodium citrate buffer, pH 6.0, using the AR98C S30M program. After blocking intrinsic peroxidase activity in 0.3% H₂O₂ in methanol for 30 minutes, The sections were incubated at 22°C as follows: (1) with 1:50 dilution of different normal serums: goat, rabbit or donkey (Jackson ImmunoResearch Laboratories, West Grove, USA) in phosphate buffer containing 0.1% bovine serum albumin (Sigma) for 30 minutes (2) the primary antibody (table 3), (3) biotinylated secondary antibody (table 3) and (4) avidin-biotin-peroxidase complex. The sites of peroxidase binding were revealed by incubation in 0.006% H₂O₂ and 3, 3’-diaminobenzidine tetrahydrochloride (DAB, Sigma) at 22°C for 1 to 10 minutes in darkness. One of the sections on each slide was counterstained with Harris hematoxylin for one minute. The sections were dehydrated in a graded ethanol series, cleared in xylene, and mounted in synthetic mounting medium (Diatex, Becker Industrifärg AB, Märsta, Sweden).

Purified nonimmune normal IgG or normal monoclonal IgG₅ (e. g. IgG₁ or IgG₂α) of the same subtype but with irrelevant specificity was used instead of and at the same concentration as the primary antibodies as a negative staining control. Three osteoclastoma samples served as positive controls in staining (kindly provided by Dr. Mika Hukkanen). Both the negative staining and positive sample controls confirmed the specificity of immunohistochemical staining results.

7. 3. 2 Indirect immunofluorescence staining

PBMCs cultured on coverslips for 1, 3, 7, or 14 days with or without rhM-CSF and rhRANKL were washed, fixed in 3% paraformaldehyde in PBS for 20 minutes, and incubated in: (1) the primary antibody (table 3) or as a negative control with purified normal IgG for 30 minutes; and (2) immunofluorescens, fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) conjugated secondary antibody (table 3) for 30 minutes. Nuclei were stained for 5 minutes in 5 µg/ml of 4’, 6-diamidino-2-phenylindole (DAPI) before mounting.

7. 3. 3 Immunofluorescence double staining

After deparafffinization 6 μm thick tissue sections were incubated for 30 minutes in 0.01 M antigen retrieval sodium citrate buffer, pH 6.0, in MicroMED T/T Mega Laboratory Microwave Systems (Milestone) using the AR98C S30M program. Nonspecific binding sites of were blocked with normal goat, rabbit or donkey serum followed by incubation in: (1) a mixture primary antibody (table 3) and CD68 (as a monocyte/macrophage marker), overnight at 4°C; and (2) a mixture of FITC conjugated and TRITC conjugated Secondary antibodies (table 3) for one hour at
22°C. Purified normal goat or rabbit IgG at the same concentration as and instead of the specific primary antibodies was used as the negative staining control. All incubations were done at 22°C if not otherwise stated. Slides were air-dried and mounted in Vectashield (Vector, Burlingame, USA) and kept in the dark at 4°C.

For PIV2 infection, cells were cultured on coverslips for 2, 24 or 72 hours, washed in PBS, fixed in acetone for 20 minutes at -20°C and incubated in 1) a mixture of ADAM8, 9 or 12 antibody with FITC-conjugated monoclonal mouse anti-parainfluenza 2 (Chemicon, Temecula, USA) for 30 minutes and 2) immunofluorescence-labeled secondary goat anti-rabbit or donkey anti-goat antibody for 30 minutes. Coverslips were mounted in Vectashield Mounting Medium containing 4', 6-diamidino-2-phenylindole (DAPI, Vector).

Table 4. Primary antibodies of this study and some of their characteristics

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Description</th>
<th>Source</th>
<th>Manufacturer</th>
<th>Concentration (µg/ml)</th>
<th>Secondary antibodies</th>
<th>Article</th>
</tr>
</thead>
<tbody>
<tr>
<td>hADAM8</td>
<td>Affinity purified</td>
<td>Rabbit</td>
<td>Cedarlane Lab, Hornby, Ontario, Canada</td>
<td>1</td>
<td>Alexa Fluor 594-labeled goat anti-rabbit IgG</td>
<td>IV</td>
</tr>
<tr>
<td>hADAM9</td>
<td>Affinity purified, polyclonal</td>
<td>Rabbit</td>
<td>Triple Point Biologics, Forest Grove, USA</td>
<td>1</td>
<td>Biotinylated goat anti-rabbit IgG</td>
<td>II</td>
</tr>
<tr>
<td>hADAM12</td>
<td>Affinity purified, polyclonal</td>
<td>Goat</td>
<td>Santa Cruz Biotechnology, Santa Cruz, USA</td>
<td>1</td>
<td>Biotinylated rabbit anti-goat IgG</td>
<td>I</td>
</tr>
<tr>
<td>hADAM12</td>
<td>Polyclonal antiserum</td>
<td>Rabbit</td>
<td>Gift from Dr. Ulla M. Wewer</td>
<td>1</td>
<td>Alexa Fluor 488-conjugated goat anti-rabbit IgG</td>
<td>I</td>
</tr>
<tr>
<td>hCathepsin K</td>
<td>Affinity purified, polyclonal</td>
<td>Goat</td>
<td>Santa Cruz</td>
<td>1</td>
<td>Biotinylated rabbit anti-goat IgG</td>
<td>III</td>
</tr>
<tr>
<td>hCD68</td>
<td>Monoclonal IgG₁</td>
<td>Mouse</td>
<td>NeoMarkers, Freemont, CA, USA</td>
<td>0.1–0.5</td>
<td>TRITC-conjugated donkey anti-mouse IgG</td>
<td>I, II</td>
</tr>
<tr>
<td>hM-CSF</td>
<td>Affinity purified</td>
<td>Mouse</td>
<td>Biogenesis, Poole, UK</td>
<td>1</td>
<td>Biotinylated horse anti-mouse IgG</td>
<td>I</td>
</tr>
<tr>
<td>hMMP-1</td>
<td>Monoclonal IgG₂α</td>
<td>Mouse</td>
<td>Chemicon, Temecula, USA</td>
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<tr>
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<tr>
<td>hRANKL</td>
<td>Monoclonal IgG₂β</td>
<td>Mouse</td>
<td>R&amp;D</td>
<td>1</td>
<td>Biotinylated rabbit anti-goat IgG</td>
<td>I</td>
</tr>
</tbody>
</table>

7.4 In situ hybridization (ISH)

Messenger RNA was isolated from total RNA. cDNA was synthesized using reverse transcriptase and oligo (dT)₁₂-₁₈. The primers were as follows ADAM 12: Sense 5'- ACA ATG CGC AGC TTG TCA GT -3'; Antisense 5'- TGA TGC AGC CTC CTT TCT CA –3'; ADAM9: Sense 5'- GCA TTT GTG GGA ACA GTG TG -3'; Antisense 5'- CCA GCG TCC ACC AAC TTA TT –
3’. Sequence similarities were searched using the NCBI Blast 2 program. Polymerase chain reaction (PCR) amplification of 5 ng cDNA samples was performed using 0.4 µM target-specific primers, AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), and a thermal cycler (RoboCycler 40; Stratagene, La Jolla, USA) for 40 cycles. The identity of the product was verified by sequencing (QiAquick; Qiagen, Valencia, USA) using an automated Applied Biosystems 373A sequencer (Applied Biosystems). The PCR products were cloned using the TOPO TA cloning kit (Invitrogen). After purifying the recombinant vector using a NucleoSpin plasmid kit (Macherey-Nagel, Düren, Germany), the automated sequencer verified the orientation of the acquired antisense and sense sequences using M13 reverse or forward primers. After linearizing the plasmid using restriction enzymes Not I or Hind III, DNA templates were transcribed to 237 bp single-strand antisense RNA probe using SP6 RNA polymerase or a sense RNA control probe using T7 RNA polymerase. RNA was labeled using digoxigenin-conjugated UTP (DIG RNA labeling kit; Roche Diagnostics GmbH, Mannheim, Germany). A dilution series of digoxigenin-labeled RNA applied to nitrocellulose membrane confirmed the efficiency of RNA labeling.

Frozen tissue sections were fixed in 4% paraformaldehyde at 4°C for 15 minutes, permeabilized for 30 minutes at 37°C in 100 mM Tris–HCl, 50 mM EDTA, pH 8.0, containing 5 µg/ml RNase-free proteinase K, and dipped in 0.1 M triethanolamine diluted 1:400 (v/v) in acetic anhydride, pH 8.0. To block non-specific hybridization, sections were prehybridized with hybridization buffer containing 100 µg/ml denatured salmon sperm DNA for one hour at 57°C in humidified chambers. After blocking sections were hybridized with 1–2 ng/µl digoxigenin-labelled RNA probe in humidified chambers at 57°C overnight. Following stringent post-hybridization washes, single-stranded (unbound) RNA probe was degraded at 37°C for 30 minutes using 20 µg/ml RNase-A (Roche). Sections were blocked in normal sheep serum at 4°C overnight. Alkaline phosphatase-conjugated sheep anti-digoxigenin-Fab (Roche) was added and colour was developed using 5-bromo-4-chloro-3-indolylphosphate (BCIP; Promega, Madison, USA) substrate and nitroblue tetrazolium (NBT; Promega) colour. 1 % methylene green was used as a counterstain before mounting.

7. 5 Polymethylmethacrylate particle uptake

Using 500-grid SiC paper, polymethylmethacrylate (PMMA, bone cement) particles containing BaSO₄ were produced from a PMMA block. These particles were preserved in a concentration of 1.3 mg/ml in 99.5% ethanol until use in cell stimulation experiments. The diameter of the particles was 0.6–2 µm characterized using a NICOMPTM 370 (Particle Sizer Systems, Santa Barbara, USA) submicron particle sizer based on dynamic light scattering using particles suspended in solvent. Before stimulation, PMMA particles were sonicated using ultrasound for dispersal and cleaning with a Vibra Cell™ processor (Sonics & Materials, Newtown, USA) with 2 second intervals (amplitude 80%, output 150 watts). Adherent monocytes were stimulated for 24 or 72 hours using 25 ng/ml of rhM-CSF alone or together with 40 ng/ml of rhRANKL in the presence or absence of 1 x 10⁶/ml PMMA particles. Non-stimulated resting cells were used as a control.

7. 6 Bone resorption assay

In vitro bone resorption assay was performed first using PBMCs stimulated with M-CSF and RANKL for 7 days to induce osteoclast differentiation (see 7.2.1). Then cells were detached with trypsin treatment followed by gentle scraping and transferred to 12-well plates containing dentine slice (size 5 x 5 mmm, Immunodiaonostic System, Boldon, UK) at the cell density of 4x10⁵ / dentine slice. After seven days incubation with M-CSF and RANKL, the cells were brushed away
and the resorption pits were visualized with toluidine blue. Samples were inspected using a Leica TCS SP2 confocal microscope (detailed in Konttinen et al. 2001).

7. 7 Western blotting

Tissue samples were homogenized in an ice-bath using sonication (Vibra-Cell 501; Sonics & Materials, Danbury, CT, USA) in RIPA buffer (RIPA buffer set; Boehringer Mannheim, Indianapolis, USA) for 15 seconds and centrifuged at 21 000 g for 3 hours at 4°C. Before electrophoresis, the total protein of the sample was quantified using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, USA). Samples were boiled in sodium dodecyl sulphate (SDS) sample buffer (187.5 mM Tris HCl, pH 6.8, 6 % SDS, 30 % glycerol, 0.03 % phenol red, and 125 mM dithiothreitol, diluted 1:3; New England Biolabs, Beverly, USA) and adjusted to 100 µg per lane. After 10% SDS–polyacrylamide gel electrophoresis, the gels were equilibrated and blotted onto nitrocellulose membrane (Bio-Rad Laboratories). The membranes were incubated at room temperature in (1) 3% bovine serum albumin (Sigma) in Tris-buffered saline blocking solution overnight, (2) the primary antibody (table 3) for 60 minutes, and (3) alkaline phosphatase-conjugated secondary antibody (Bio-Rad Laboratories) for 60 minutes. Between the steps, the membranes were washed in 0.1% Tween 20 in 50 mM Tris, 0.9% NaCl, pH 7.5 (Tris-buffered saline). Colour reaction was developed for 15 minutes using BCIP and NBT (Alkaline Phosphatase Conjugate Substrate Kit, Bio-Rad Laboratories).

Human peripheral blood monocytes were costimulated with rhM-CSF and rhRANKL for 1, 3, 7, or 14 days in 6-well plates and then lysed with 200 µl Passive Lysis Buffer (Promega). Cell debris was removed by centrifugation at 10,000 g for 10 minutes at 4°C and supernatant was stored at –70°C. Before electrophoresis, the total protein of the sample was quantified using Bio-Rad protein assay (Bio-Rad Laboratories), the sample was boiled in SDS containing sample buffer (New England Biolabs) at 98°C for 5 min and 75 µg protein was loaded per lane. After SDS-polyacrylamide gel electrophoresis, samples were blotted onto nitrocellulose membrane (Bio-Rad). Membranes were stained at room temperature as mentioned above.

7. 8 Flow cytometry of monocyte/macrophages

Adherent cells were stimulated either for 24 h or 72 h using 25 ng/ml rhM-CSF (R&D) alone or together with 40 ng/ml rhRANKL (Alexis) in the presence or absence of 1 x 10^6/ml PMMA particles. They were gently scraped and fixed with 4% w/v paraformaldehyde for 30 minutes at 4°C and permeabilized with Perm/Wash buffer (Becton Dickinson, San Jose, CA). 2 x 10^5 cells were incubated in (1) the primary antibody/antibodies (table 3) at 4°C for 30 minutes. (2) incubated for 60 minutes in dark with immunofluorescence conjugated secondary antibody/antibodies (table 3). The stained cells were analysed with a FACScan analyser using CellQuest software (Becton Dickinson). The level of non-specific binding was determined using isotype-matched antibodies of irrelevant specificity.

7. 9 Measurement of degraded collagens in tissues

Degraded collagen was measured as described in detail elsewhere (Bank et al. 1997). Briefly, tissue block around 3 x 3 x 2 mm was incubated with 4 M guanidine chloride-hydrochloric acid in incubation buffer of 0.1 M Tris-HCl, pH 7.3 containing 1mM iodoacetamide, 1 mM EDTA and 10 µg/ml pepstatin-A for 48 hours at 4°C to remove proteoglycans. After washing with incubation buffer 3 times for 3-6 hours at 4°C, followed by incubation in 1 mg/ml α-chymotrypsin to digest denatured collagen in the insoluble matrix. After hydrolyzing the supernatant and pellet in 6 N
HCl at 110°C for 20-24 hours, the hydrolyzates were dried overnight and to residue HCl, samples were reconstituted with distilled water and dried again. Finally, the samples were dissolved in 1 ml of 0.1 M borate buffer, pH 9.5 undiluted for supernatant (hydrolyzates of supernatants) or diluted 1:250 for non-soluble matrix (hydrolyzates of pellet) ready for amino acid analysis.

200 μl samples were mixed with 25 μl 30 mg/ml o-phthaldialdehyde containing acetone and 1.5 % (v/v) β-mercaptoethanol for one minute at room temperature. 25 μl of 80 mg/ml iodoacetamide in acetone was added for at least 30 seconds at room temperature to remove excess β-mercaptoethanol by precipitation. After derivatization with 9-fluorenylmethyl chloroformate, hydroxyproline and proline were extracted with diethyl ether and dissolved in solvent A (20 mM sodium citrate containing 5 mM tetramethylammonium chloride and 0.01 % NaN₃, pH 2.85). Elution was performed in two isocratic steps using 60 % and 25 % solvent A with 40 % and 75 % solvent B (acetonitrile) with 1 ml/min flow rate, on-line monitoring at excitation wavelength of 254 nm and emission wavelength of 630 nm at room temperature. The percentage of total collagen present in tissue was calculated and described as the amount of degraded collagen. degraded collagen % = hydroxyproline content in supernatant digested by α-chymotrypsin × 100% / (hydroxyproline content in supernatant digested with α-chymotrypsin + hydroxyproline content of digested plug).

7. 10 Image analyse

Specimens were inspected using 200 x magnification and Leitz Diaplan-microscope (Leitz, Wetzlar, Germany) or Olympus motorized revolving AX 70 system microscope, which is coupled to 12-bit PC digitalcamera (SensiCam, Kelheim, Germany). Pictures were analysed semiautomatically using AnalySIS Pro 3.2 Image analys- and processing datasystem (Soft Analysis System GmbH, Münster, Germany). The images were analyzed morphometrically after adjusting illumination and focus. The whole section area was analyzed. The results of quantitative assessment are expressed as percentage of positively staining area of the total tissue area.

7. 11 Statistical analysis

The results are mainly expressed as mean and standard error of the mean (SEM). Some data expressed as range and median value. Wilk's W test was performed for checking normality of all data. The t-test was performed for normally distributed data between groups, while Wilcoxon's test for skewed data. Probability values less than 0.05 were considered statistically significant. These statistical calculations were done using statistical software BMDP-PC version 7.01 (BMDP Statistical Software, Cork, Ireland).

7. 12 Ethical aspects

The work had been accepted by the ethical committee Helsinki University Hospital (HUS). Helsinki and Vancouver declarations were followed.
8. RESULTS

8.1 M-CSF and RANKL in interface membrane

Immunohistochemical analysis revealed intense M-CSF and RANKL immunoreactivity in many cells of the synovial membrane-like interface membrane around loosened THR implants. M-CSF was mostly found in macrophage-like cells and multinuclear giant cells, which often contained phagocytosed cytoplasmic foreign bodies, whereas RANKL was found both in fibroblast- and macrophage-like cells. In contrast to the relatively extensive and strong immunoreactivity in revision total hip replacement samples, M-CSF and RANKL containing cells were relatively few and staining was weak in control synovial membranes obtained from primary total hip replacements. For practical and ethical reasons it is not easy to obtain implant capsule tissue control samples from well-fixed total hip implants, although occasionally well fixed implants are revised because of periprosthetic fracture or mechanical failure including dislocations, malpositions and instability, etc. The absence of appropriate control samples is one of the limitations of these studies. In this study, we used synovial membrane obtained from patients with osteoarthritis undergoing primary THR as control tissue, because inflammation in such tissue is often mild or minimal. Traumatic synovial membrane would probably have been an even better control. However, the clear difference between interface membrane and membrane from osteoarthritis membrane clearly suggests an active role for M-CSF and RANKL in aseptic loosening. Negative staining controls were negative and confirmed the specificity of the staining.

8.2 ADAMs expression and osteoclast formation

8.2.1 ADAM9 and ADAM12 in interface tissue

Several ADAM12 positive cells were detected in synovial membrane-like interface tissue, whereas fibrous area filled with collagen fibers and fibroblast-like cells did not stain for ADAM12. Blood vessels occasionally stained for ADAM12. All multinuclear foreign body giant cells were ADAM12 positive. In double immunofluorescence staining, CD68 marker used to detect macrophages and multinuclear giant cells in the synovial membrane-like interface tissue around loosened THR implants, all CD68 positive cells were also ADAM12 positive. In addition, mononuclear ADAM12 positive cells were often detected closely to multinuclear ADAM12 positive cells. Negative staining controls and positive sample controls confirmed the staining results. Negative control experiments performed using consecutive sections did not show any labeling. In positive osteoclastoma sample controls (Tian et al. 2002) the percentage of ADAM12 positive cells (74 ± 3%) ranks the highest, while osteoblasts, osteocytes, and chondrocytes were ADAM12 negative. Both macrophage-like and foreign-body giant cells were ADAM12 positive in the interface membrane, whereas control membranes contained only a few ADAM12 positive macrophage-like cells and no ADAM 12 positive foreign-body giant cells. Morphometrically, the comparison between the percentage of ADAM12 positive cells in revision THR and in primary THR samples was statistically significant (53 ± 2% vs. 5 ± 1%, p<0.001). Moreover, the intensity of cellular ADAM12 staining in revision THR samples was higher than in primary THR samples.

Immunohistochemical staining was used to localize ADAM9 and its cellular sources in interface membrane tissue samples. The staining intensity varied in different samples and different regions. Intense ADAM9 immunoreactivity was detected in all synovial membrane-like peri-implant interface tissue samples around loosened implants. The inflammatory, cell-rich areas contained several ADAM9 positive macrophage-like cells, whereas such cells were much rarer in the deep fibrotic tissue areas which lack of ADAM9 positive signal. In particular, the synovial lining-like
layer facing the pseudosynovial fluid always exhibited intense staining in interface tissue samples. All multinuclear foreign body giant cells were ADAM9 immunoreactive. Mononuclear ADAM9 positive cells were often close to ADAM9 positive multinuclear cells or in small aggregates in intimate contact with each other. Cellular ADAM9 staining was stronger in revision THR than in control synovial membrane samples from the primary THR which exhibited weak immunoreactivity for ADAM9, and restricted to certain areas. The percentage area of ADAM9 positive immunoreactivity was measured because of some extracellular ADAM9 staining. The percentage of ADAM9 positive area in revision THR was different from that of the primary THR samples (37.6 ± 5.1% vs. 5.2 ± 0.8%, p=0.002). Staining controls produced by using purified non-immunized rabbit IgG at the same concentration as and instead of specific primary anti-ADAM9 IgG were almost totally negative.

The total overlap between ADAM9 immunoreactivity with CD68 positive cells was disclosed using TRITC and FITC double staining. The close spatial relationships were found between ADAM9 positive mononuclear cells and ADAM9 positive multinuclear giant cells and also between CD68 positive mononuclear cells and CD68 positive multinuclear giant cells. All ADAM9 positive cells were also CD68 positive in interface tissue. In control osteoarthritic synovial membrane samples ADAM9 immunoreactivity was usually weak and restricted to certain areas, while all CD68 positive macrophages were also ADAM9 positive in double staining. A 50 KDa band in the Western blots was ADAM9 immunoreactive in tissue extract samples of revision THR but was not found in samples from primary THRs.

In situ hybridization demonstrated ADAM12 messenger RNA in macrophage-like cells and in multinuclear foreign body giant cells. Furthermore, the synovial lining-like layer and some blood vessels contained ADAM12 messenger RNA, whereas fibroblast-like cells were not labeled. Then in the consecutive tissue labeled using sense RNA probe showing negative labeling confirmed the specificity of the labeling.

In situ hybridization of ADAM9 messenger RNA using DIG-labeled ADAM9 cRNA probe detected ADAM9 messenger RNA in macrophage-like cells and multinuclear giant cells. ADAM9 messenger RNA was found in all synovial membrane-like interface tissue samples around loosened hip implants. The lining-like layers and macrophage-rich sublining areas showed strong signal, whereas ADAM9 transcript positive cells more rarely appeared in the deep stroma of the interface tissue samples. Similarly to immunostaining, ADAM9 messenger RNA positive mononuclear cells were often seen in close contact with ADAM9 mRNA positive multinuclear cells. The negative sense RNA probe in the consecutive tissue sections confirmed the specificity of the labeling. In tissue controls ADAM9 mRNA was weakly detected and restricted in synovial lining and sublining areas of the samples obtained from primary THR operations. This indicates ADAM9 transcripts much less in the controls.

8. 2. 2 ADAMs in M-CSF and RANKL stimulated osteoclast formation assay

Peripheral blood mononuclear cells stimulated with M-CSF and RANKL and adherent to coverslips were stained to analyze fusion process over time. Those cells which had not been stimulated with M-CSF and RANKL showed weak staining of ADAM9. One day after M-CSF and RANKL costimulation the ADAM9 staining was still weak. Cultured cells were large, but still mononuclear and ADAM9 expression was stronger compared to day 1, widespread and slightly granular at culture day 3. At culture day 7, several bi- or multinuclear cells had appeared and ADAM9 staining was quite strong. At culture day 14, most of ADAM9 positive cells were multinuclear giant cells and the percentage of mononuclear ADAM9 positive cells declined.
ADAM9 staining showed strong and non-homogenous cytoplasmic granular patches.

According to FACS analysis, all ADAM12 positive monocytes cultured for one day in the presence of M-CSF and RANKL were also CD14 positive. These FACS analysis results were similar to those of resting peripheral blood monocytes without M-CSF and RANKL stimulation. In vitro stimulation of peripheral blood monocytes with M-CSF and RANKL for a short one-day time did not seem to increase significantly the number of ADAM12 molecules per cell. At culture day 1, ADAM12 was detected usually as one cytoplasmic patch in M-CSF and RANKL costimulated human peripheral blood monocytes. At culture day 3, all cells were large, showing widespread cytoplasmic staining, but almost all cells were still mononuclear. At culture day 7, the cells were very large and began to fuse, showing several bi- and multinuclear cells with relatively strong cytoplasmic granular ADAM12 staining. At culture day 14, the the percentage of multinuclear giant cells increased, while the percentage of mononuclear cells decreased (Figure 7). According to morphometric analysis, 26 % of the cells were binuclear and 12 % multinuclear at culture day 7 and 14 % of the cells were binuclear and 66 % multinuclear at culture day 14. These results imply that fusion started at day 3. When transferred to dentin slices at culture day 7 and cultured for seven additional days, these cells at day 14 had formed resorption pits indicating the capability of bone resorption in vitro as shown before (Mandelin et al. 2005).

As monocyte/macrophages are both adherent and long living cells, the analysis of ADAM 9 and ADAM 12 staining patterns over time could be performed using adherent cells cultured till 14 days on coverslips. Human peripheral blood monocytes were enriched and stimulated with M-CSF and RANKL in vitro to induce osteoclast-like cell formation. Such costimulation led to characteristic time-dependent phenotypical changes of the cell size, number of nuclei and ADAM staining pattern. These changes to a cytoplasmic granular staining pattern of the bi- and multinuclear cells observed at days 3 and 7 of stimulation and further to even more extensive granular and patchy cytoplasmic staining pattern by day 14 in multinuclear cells.

PBMCs were also cultured both in the presence and in the absence of PMMA particles were ADAM9 positive at day 1 according to flow cytometric analysis. At culture day 3, the ADAM9 staining intensity increased with a clear change in staining over time, while non-stimulated resting cells were ADAM9 negative. With or without M-CSF and RANKL did not significantly affect the result of PMMA stimulation.

Western blots of cell extracts were prepared from M-CSF and RANKL costimulated human peripheral blood monocyte cultures at culture day 1, 3, 7 and 14. The synthesis of the latent 90 kD
ADAM12 had apparently increased upon the stimulation after 3 days of M-CSF and RANKL costimulation and remained high until the end of the 14-day culture. In addition to the increased expression, a weak 60 kD band was detected, most clearly in cells stimulated for 3 days. These studies demonstrated that the 60 kD ADAM band represents, as assessed by its immunoreactivity and size, metalloproteinase-cleaved, fusion-active ADAM 12.

8. 2. 3 ADAMs in the PIV2 infected host cells

At 2 hours, PIV2 antigens were not yet found in infected GMK cells and also ADAM8 and ADAM9 were negative (Figure 8, A, B). At culture day 1, PIV2 antigens were seen in infected GMK cells. In addition, infected and some uninfected GMK cells were ADAM9 positive (Figure 8, C), but negative for ADAM8. At culture day 3 PIV2 had infected most GMK cells and had caused cytopathic effects so that large multinucleated syncytia were seen. Staining of ADAM9 positive multinuclear giant cells was relatively strong (Figure 8, D) but no ADAM8 positive cells were seen.

**Figure 8** Immunofluorescence double staining of ADAM9 and PIV2 in PIV2 virus infected GMK cell cultures at day 0 (panel A, B), 1 (panel C) and 3 (panel D). ADAM9 staining is shown in red and PIV2 in green, together with the blue nuclear counterstain. At day 0, PIV2 antigens were not yet seen inside the cells (A) and ADAM9 staining was negative (B); (C, D) overlay of double staining of ADAM9 and PIV2 at culture day 1 and day 3, respectively. Bar = 10 μm.
PIV2 were not detected in the HSG cell cultures in the presence with PIV2, so HSG cells were not infected PIV2 at not all. While PIV2 infected human HSY cells on coverslips were stained to analyze the cellular staining pattern during different time points of infection. The uninfected cells showed very weak ADAM8 signal. At culture day 0, PIV2 had not yet infected HSY cells and ADAM8 showed weak staining. At culture day 1, PIV2 was seen in HSY cells, where numbers of cytoplasmic patches of ADAM8. At culture day 3, PIV2 was seen in most HSY cells and the numbers of adherent cells were lower, because PIV2 infected and multinucleated cells were nonadherent. ADAM8 positive, multinuclear giant cells stained relatively strongly with a non-homogenous granular and widespread patchy cytoplasmic staining.

8. 3 Collagen degradation analysis

Three different collagenolytic enzymes, MMP-1, MMP-13 and cathepsin K were examined both in the control synovial membrane from trauma patients and in the interface membrane around loosened total hip replacement implants (Table 5). The numbers of cells positive for all three proteins were higher in the interface membrane than in the control synovial membranes, as follows: MMP-1 (range 110-340, median value 325.5 vs. 48-80, median value 62), MMP-13 (range 74-261 median value 139 vs. 12-52 value 23) and cathepsin K (range 58-157 median value 119 vs. 5-23 median value 15). The approximate fold-differences of the positive cell numbers between patient and control are 5 folds for MMP-1, 5 folds for MMP-13 and 7 folds for cathepsin K. Compared to 9.2%-13% (median value 13%) degradation in control synovial membrane from traumatic patients, collagen in interface membrane around aseptically loosened totally replaced hip implants was degraded to 18%-34% (median value 21%). The correlations between collagen degradation and the number of cells expressing these proteinases were calculated. In aseptic loosening correlations with the degree of collagen degraded were for MMP-1 (r = 0.88, P = 0.002), MMP-13 (r = 0.92, P = 0.001) and cathepsin K (r = 0.98, P < 0.001). In contrast, in normal synovial membrane these correlations were far from significant although MMP-1 come close to statical significant (r = 0.86, P = 0.062). The corresponding values for MMP-13 (r = 0.63, P = 0.26) and cathepsin K (r = 0.009, P = 0.99) were not significant.

Table 5. Comparison of three collagenolytic enzymes in patient samples with controls
9. DISCUSSION

9.1 ADAMs expression and osteoclast formation in interface tissue

M-CSF was first described and purified from mouse yolk sac-conditioned medium (Johnson et al. 1978), while RANKL was first found on the surface of activated T-cells (Anderson et al. 1997, Wong et al. 1997). It is known that the cytokine combination of M-CSF and RANKL is necessary to induce hematopoietic cells or monocytes to fuse to form morphological and functionally typical osteoclasts in vitro (Quinn et al. 1998). Therefore, we aimed to confirm the presence of both M-CSF and RANKL in our revision samples. The presence of M-CSF and RANKL in interface tissue seems to confirm the molecular machinery able to induce and stimulate progenitor cell fusion to foreign body giant cells and osteoclasts which both are present at this scene of the aseptic loosening process. M-CSF and RANKL are also essential to podosome rearrangement and for the intracellular signaling pathway that induced cytoskeletal and motility changes in osteoclasts which lead to localized bone resorption and osteolysis (Pfaff and Jurdic 2001, Grey et al. 2000).

Aseptic loosening of THR implant could be considered as macrophage-driven chronic inflammation or macrophage-driven synovitis, which is characterized by macrophage accumulation and activation. Because CD68-positive synovial tissue macrophages were all ADAM12 positive, as revealed using immunofluorescence double staining, and over half of the cells in the synovial membrane-like interface tissue were ADAM12 positive macrophage-like cells, compared to only 5% in the corresponding control samples from osteoarthritic synovial membranes, these results showed how intense the local accumulation of macrophages is. Normally, monocytes transmigrate to the interface membrane from the circulation, but in foreign body reaction this might be amplified further by M-CSF which drive macrophages to proliferate locally. On the other hand, T-lymphocyte responses could be able to contribute to pathogenesis of aseptic loosening as has been suggested in particular for the metal-to-metal prosthesis (Weyand et al. 1998, Farber et al. 2001, Hallab et al. 2005). Metal ions are produced from electrochemical degradation (corrosion) which can as haptons bind to proteins making them allergens. However, the most frequently used prosthesis type is metal-to-UHMWPE, which produces mainly UHMWPE wear particles. Aseptic loosening of this type of prosthesis is often characterized by particle disease and macrophage dominance was demonstrated using ADAM12 labeling showing above. Metal-on-metal implants were not studied in these case series.

Based on the results above, it is apparent that ADAM12 mRNA- and protein-positive mononuclear cells were often seen in close spatial relationship to each other. ADAMs has multipurpose domain structure consisting of the N-terminal end prodomain, which is followed by a metalloproteinase domain and a disintegrin domain. This is the origin for the name of this whole family of molecules. As a transmembrane molecule containing extracellular metalloproteinase domain ADAM12 represents cell membrane-associated proteolytic potential enabling cell-mediated extracellular matrix remodeling (Le et al. 2003). This might contribute to monocyte/macrophage transmigration so that they can move to interface tissues. In accordance with this hypothesis, our results showed that all monocytes and macrophages seemed to be ADAM12-positive. The disintegrin domain of ADAM12 is involved in binding of integrin receptors, therefore, it might be involved in a non-RGD-dependent manner in cell–extracellular matrix and cell–cell attachment and signaling. Both cellular attachment and formation of polykaryons are important processes in aseptic loosening. Both M-CSF and RANKL are abundant
in interface tissue as shown above. This could be implied that these two factors drive prefusion macrophages to form polykaryons with the help of ADAMs.

After metalloproteinase and disintegrin domains in ADAM proteins follow the cysteine-rich domain and epidermal growth factor-like domain, which may be involved in some membrane fusion and cell fusion reactions (Becherer et al. 2003). The intracellular, cytoplasmic C-terminal tail is highly variable both in length and sequence. The interaction of the cytoplasmic domain of ADAM12 with intracellular actin cytoskeleton has the potential to control fusion process by transducing signals and by regulating the cytoskeleton (Cao et al. 2001).

ADAM12 plays a role at multiple levels of cell-to-cell fusion process. Both the immunohistochemical staining results of the M-CSF and RANKL costimulated cultured human monocytes and Western blotting of cell extracts demonstrated the increased expression of the prodomain cleaved ADAM12, with an approximately molecular weight of 92 kD. However, the full length 92 kD ADAM12 can not catalyze fusion. This requires the removal of the metalloproteinase domain, which caps the full-length latent ADAM12 thus preventing these fusion-promoting activities (Schlomann et al. 2002, Yagami-Hiromasa et al. 1995). Quite corresponding with Western blotting of cell extracts, in particularly, at day 3 before the peak of cell fusion some of ADAM12 was found also in the 60 kD area. This band corresponds to the metalloproteinase-cleaved, fusion-active form of ADAM12 based on its molecular weight and its time of appearance. This time point just before the monocytes start to fuse and form multinucleated cells corresponds with the conversion of the full length ADAM12 into its metalloproteinase-cleaved 60 kD active form. This is also accordance with results from in vitro myogenesis studies in which ADAM12 mRNA increased before fusion and decreased during the completed cell fusion process. The progressive upregulation of the ADAM12 gene until day 8 in cultures of human peripheral blood monocytes undergoing fusion followed by a dramatic decrease of its mRNA expression at day 9 has been reported previously (Verrier et al. 2004). This suggests a role for ADAM12 in the early stage of the human osteoclast formation.

Our study is the first to demonstrate a disintegrin and a metalloproteinase ADAM9 in the interface tissues of revision THR patients. Both macrophage-like and foreign body giant cells were ADAM9 positive in the interface membrane. Double immunofluorescence labelling of ADAM9 and CD 68 revealed the expression of ADAM9 in all macrophages and monocytes. ADAM9 positive mononuclear cells represent prefusion cells which locate in close contact with multinuclear cells. ADAM9 expression in revision THR tissues was also confirmed by Western blot analysis. However, the lack of ADAM9 band in Western blots of control synovium membrane samples is in a discrepancy with our immunohistochemical ADAM9 staining of such samples. This may be explained so that the ADAM9 concentration in the extracts remains below the detection level of the Western blot method while the staining still shows few positive cells. Immunohistochemistry can be used to show individual positive cells even among hundreds or thousands of other negative cells.

ADAM9 has a complex and multifunctional domain structure and seems to play multiple roles during cell fusion. It contains a putative fusion peptide sequence, which like viral fusion peptides, may participate in membrane fusion via its cysteine-rich domain (Blobel et al. 1992). Some studies have investigated the fusogenic activity of this kind of synthetic peptides binding with liposomes (Martin et al. 2000). Further studies using overexpression or mutants of cysteine-rich domain would elucidate ADAM9 structure and function relate to the process of cell fusion.
In both ADAM9 and ADAM12 studies, in interface membrane mRNA and protein positive mononuclear cells were often seen in a close spatial relationship to each other. As interface tissue was apparently also a rich source of M-CSF and RANKL, it is possible that these two factors drive prefusion macrophages to form polykaryons. Expression of ADAMs in both osteoclast precursors and mature osteoclasts indicates that these proteins are involved in cell fusion and osteoclast activity (Verrier et al. 2004). As fusion molecules, ADAM9 and ADAM12 overexpress in the trans-membrane when macrophages develop abundant interdigitations in their adjacent plasma membranes. Both ADAM9 and ADAM12 contain a stretch of hydrophobic amino acids, known as a fusion peptide, which penetrates adjacent cells to break the lipid bilayer of the cells. Then they undergo a conformational change and propel the plasma membrane close enough to trigger to open and fuse the membranes. Once fusion of the membrane structure has been initiated, the cytoplasms are integrated. Studies on the biochemical regulation and biological modulation of ADAMs are in progress. Intracellular, pericellular and gene level regulation control the activities of ADAMs. After cleavage of the prodomain by a furin-peptidase in the trans-Golgi, the mature form of human ADAM12 is stored intracellularly and has a potential to be translocated to the cell surface. It has been reported that protein kinase C epsilon induces ADAM12 translocation and expression to the cell surface (Sundberg et al. 2004). Both β1 and β3 integrins can bind to ADAM12 to regulate cell adhesion (Thodeti et al. 2005). ADAM9 expression has been shown to be regulated by intracellular reactive oxygen species and/or hydrogen peroxide which are generated in stressed cells (Sung et al. 2006). ADAM9 mRNA levels were downregulated upon treatment with interleukin-1α (Flannery et al. 1999). In interface tissue, the regulation of ADAM9 and ADAM12 may be modulated by locally accumulated macrophages in response to wear particles. ADAMs are apparently multidomain, multipurpose molecules important for monocyte/macrophage function, including fusion of these cells to multinuclear foreign-body giant cells responding to wear debris and to the formation of osteoclasts resorbing peri-implant bone in aseptic loosening of total hip replacement.

The virus-induced HSY cell fusion may represent cytopathic effect which is able to spread viral particles from infected cells to other infected or uninfected cells. Thus, this process shares some features with monocytes–macrophage forming multinuclear cells. In this study, we launched a new PIV-2 induced GMK cell and HSY cell fusion model to be able to characterize putative fusion molecules ADAM8 and ADAM9. Of these fusion molecules, ADAM9 was expressed in PIV-2 infected GMK cells and multinuclear giant cells, while ADAM8 was expressed in HSY cells and multinuclear giant cells. After several days of coculture with PIV-2, cytopathic effects were seen in form of formation of large multinucleated cells which showed poor adherence to the cell culture dish (Henrickson et al. 2003). Coculture with PIV-2 virus and infection of the GMK and HSY cells was accompanied by phenotypical changes of the cells, number of nuclei and ADAMs staining pattern. At culture day 0, PIV2 virus was not yet seen in either GMK or HSY cells and ADAM8 was only weakly expressed in HSY cells, while ADAM9 was negative in GMK cells. This was changed by culture day 1 as PIV2 antigen was seen both in GMK and HSY cells, and GMK cells showed ADAM9 positive and HSY cells showed cytoplasmic patches of ADAM8. At culture day 3, PIV-2 antigen was seen in most GMK and HSY cells and the formation of large multinucleated cells were induced so that the number of adherent cells decreased and both ADAMs staining was relatively strong with a non-homogenous granular and widespread patchy cytoplasmically staining pattern. These changes in ADAMs expression upon formation of polykaryons suggest that ADAMs may dynamically participate in the fusion process and the formation of multinuclear giant cells. This new model allows the characterizations of the molecular mechanism of the fusion proteins in the future.
9. 2 Collagen degradation as part of osteoclast function

This is the first study directly demonstrating the increased collagen degradation in the interface membrane of aseptic loosening compared to control synovial membrane from the patients with hip fracture. These measurements demonstrate enhanced remodelling and/or pathological collagen tissue destruction in interface tissue. As a synovial membrane-like interface membrane always surrounds failing implants, this interface has been considered to play a role in peri-implant tissue destruction and loosening. Interface tissue is characterized by non-autoimmune, monocyte/macrophages and fibroblasts dominated chronic foreign body inflammation (Atkins, et al. 1997, Goodman et al. 1998). It can be considered as a prototype of and an interesting model for foreign body inflammation and macrophage- and fibroblast-mediated synovitis (Hembry et al. 1995). In aseptic loosening the macrophage lineage cells are usually activated (Chun et al. 1999), but also metal hypersensitivity and lymphocyte-mediated responses have been observed in metal-on-metal hips even though they are rare and patient number was small on the reports (Weyand et al. 1998, Arora et al. 2003). Numerous studies have demonstrated various collagenases and cathepsin K in the synovial membrane-like interface membrane, whereas it is not known whether these proproteinases are activated and may overcome their endogenous enzyme inhibitors. Low pH and cathepsin K have been shown in aseptic loosening (Konttinen et al. 2001). Synovial membrane from patients with hip fracture is not a perfect control for the synovial membrane-like interface tissue, however, for ethical reasons it was not possible to take samples from implant capsules or synovial membrane-like interface tissue in well fixed implants because broken implants are extremely rare although occasionally implant capsule could be obtained for example from patients with hip dislocation. Synovial membrane from patients with hip fracture was therefore used as control to synovial membrane-like interface. Synovial membrane after hip fracture is probably slightly inflammatory, and compared to healthy tissue has probably more pro-inflammatory cytokines and proteinases (Walakovits et al. 1992). In spite of this, a statistically significant and probably biologically relevant difference was noticed between the interface and control synovial membranes.

Both MMP-1 (collagenase-1) and MMP-13 (collagenase-3) have been reported to be increased in aseptic loosening (Santavirta et al. 1993, Imai et al. 1998, Syggelos et al. 2001). It seems that in particular monocyte/macrophages form the primary source of MMPs in prosthetic interface tissue. Moreover, cathepsin K has also been described in such tissues in some cells of multinucleated giant cells and the monocyte/macrophages (Konttinen et al. 2001, Buhling et al. 2001). In these respects the present study confirms these earlier findings by demonstrating that MMP-1, MMP-13 and cathepsin K tend to increase in aseptic loosening compared to control synovial membrane. As this is the first study to measure the amount of degraded collagen in interface membrane we were able to perform a correlation analysis between the proteinase positive cells against the proportion of collagen degraded. The hypothesis was that if there is a statistically significant correlation, this would be compatible with a role of the proteinase in local collagen degradation. If there were no correlation at all, this would argue against a significant role of that proteinase in collagen degradation. In control synovial membrane these analyses disclosed no statistically significant correlations between the three proteinases analyzed and the extent of collagen degradation although MMP-1 came close to statistical significance. This might indicate that the fibroblast collagenase MMP-1 plays some role in normal extracellular collagen remodeling. The correlation between cathepsin K and extracellular collagen degradation was very low and therefore totally non-significant in the control samples. This is compatible with the intracellular, intralysosomal nature of the acidic endoproteinase cathepsin K in control samples. If cathepsin K participates in collagen matrix remodeling in these control tissues, it occurs probably intracellularly in phagolysosomes (Everts et al. 2003). According to similar reasoning in control (patients with hip
fracture) synovial membranes collagen degradation is possibly also mediated by some other enzymes, such as neutrophil-derived or mesenchymal cell-derived collagenase 2 (MMP-8) or human trypsin 2 (Hanemaaijer et al. 1997, Stenman et al. 2005) or mechanically mediated and intracellularly finished.

All proteinases analyzed in the present study were increased in aseptic loosening. Most importantly, the extent of extracellular collagen degradation was statistically significantly correlated to the number of local cells containing these collagenolytic proteinases as demonstrated in immunohistochemical staining. In this respect, it is important to note that macrophages and fibroblasts do not produce MMPs in response to storing foreign bodies. In contrast, they are soon after synthesis secreted to the extracellular space. Although cathepsin K is in part targeted to and activated in lysosomes, also it is in part a proenzyme secreted to the extracellular space via a differentially regulated process (Claveau et al. 2001, Hou et al. 2002). Therefore, our findings suggest that all these collagenolytic enzymes overcome their endogenous inhibitors, are activated to act in concert with each other and participate in pathological peri-prosthetic tissue destruction. As MMPs can not act in acidic pH and cathepsin K can not act in neutral pH, it is possible that some areas of the interface membrane are neutral and some others acidic. It is otherwise difficult to explain the highly significant correlation between these enzymes in the pathological interface membrane and lack of such correlation in almost healthy, but traumatic control synovial membrane. In healthy synovial membrane in particular MMP-1 could be responsible for normal collagen tissue remodeling. This is supported by the observation that the correlation between MMP-1 and collagen degradation was higher and more significant than that between MMP-13 and collagen degradation or cathepsin K and collagen degradation in synovial membrane controls. However, the correlation between MMP-1 and collagen degradation was not statistically significant (r = 0.86, P = 0.062). This leads to two further conclusions. This correlation, although lacking statistical significance, might still be biologically significant. Alternatively, maybe this enzyme together with some other enzyme(s) with collagenolytic potential is responsible for extracellular collagen degradation in control synovial membranes. Present findings also support the hypothesis that cathepsin K, apart from its intracellular phagolysosomal function in healthy synovial membrane, has also an extracellular role in pathological human tissue collagen degradation in the interface membrane (Kafienah et al. 1998). This is compatible with the observations demonstrating that the interface membrane is acidic to such an extent that this even leads to demineralization of peri-implant bone (Konttinen et al. 2001). However, based on our study, it is not possible to impart definite roles for MMPs and cathepsin K in this respect. For such an analysis more work e.g. applying antibodies recognizing neoeptitopes disclosed by MMP-type collagenases and cathepsin K suitable for immunohistochemistry will be necessary (Atley et al. 2000, Wu et al. 2002).

10. CONCLUSIONS

1. This study contributes to the clarification of the reaction of human body and skeleton against implant-derived foreign bodies. This is important as implants are used in ever increasing scale for treatment of primary hip osteoarthritis, developmental dysplasia of hip joint, hip fractures, post-traumatic osteoarthritis. More specifically, the study produces new information about mechanisms of aseptic loosening of total hip replacement prosthesis and points to some new potencial targets for prevention and treatment of loosening. Total hip replacement is the gold standard for treatment of advanced hip osteoarthritis refractory to conservative treatment. Foreign body inflammation seems to be the force driving local osteoclast formation and peri-implant bone
destruction. Therefore, three members of ADAM family (ADAM8, ADAM9 and ADAM12) have all tentatively been identified as important molecules in foreign body reaction. They were analyzed in interface membrane and in \textit{in vitro} cell stimulation models. We characterized their eventual presence and levels in the interface membrane in the patients and their presence and regulation in two different \textit{in vitro} models. ADAMs may mediate fusion of progenitor cells into multinuclear foreign body giant cells and osteoclasts.

2. An increased extent of collagen degradation in interface membranes in aseptically loosened hip joint implants as compared with synovial membranes from control patients was for the first time demonstrated in the present study. Collagen degradation highly significantly correlated with the number of local cells containing collagenolytic MMPs and cathepsin K was also demonstrated in this study. This may suggest that the foreign body reaction leads to increased production, secretion and activation of collagenolytic neutral and acidic proteinses which weaken periprosthetic collagenous tissue. This may contribute to loosening of the implants from host tissue and extension of the prosthetic joint space so that it to an increasing extent surrounds the loosened implant facing the synovial membrane-like lining.

3. In our second \textit{in vitro} cell stimulation model, the virus-induced target host cell fusion may help the virus to safely spread from infected to uninfected cells. It shows that PIV2 is capable to utilize host cell fusion molecules ADAMs to spread and form syncytia. The virus-HSY cell system provides a novel experimental system to study human cell-to-cell fusion and fusion molecules, such as ADAMs.

\begin{center}
\begin{tikzpicture}

\node[anchor=north west, inner sep=0] at (0,0) {\textbf{Wear particles}};

\node[anchor=north west, inner sep=0] at (0,-1) {\textbf{Macrophage activation}};

\node[anchor=north west, inner sep=0] at (0,-2.5) {\textbf{Osteoclast formation}};

\node[anchor=north west, inner sep=0] at (0,-4) {\textbf{Implant loosening}};

\draw[->] (0,-0.5) -- (0,-0.75) node[midway, above] {RANKL, M-CSF, other cytokines and chemokines}
\draw[->] (0,-0.75) -- (0,-1) node[midway, above] {Fusion molecules, ADAM8, 9 12}
\draw[->] (0,-1) -- (0,-1.5) node[midway, above] {MMPs and cathepsin K}
\draw[->] (0,-1.5) -- (0,-2) node[midway, above] {IL-1, IL-6, TNF-\(\alpha\), GM-CSF, PGE2, M-CSF, TGF-\(\alpha\) etc.}
\draw[->] (0,-2) -- (0,-2.5) node[midway, above] {}
\draw[->] (0,-2.5) -- (0,-3) node[midway, above] {}
\end{tikzpicture}
\end{center}

\textbf{Figure 9.} Cascade of wear particle induced macrophage activation, osteoclast formation and implant loosening.
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Ma Guofeng
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Terminology

**Arthrography** The radiographic visualization of a joint (as the hip or shoulder) after the injection of a radiopaque substance. 22

**Arthroplasty** Plastic surgery of a joint (as the hip or knee), the operative formation or restoration of a joint. 9, 11

**Biocompatibility** The inherent ability of a material to appropriately interact with the host in a specific application. Biocompatibility may be further subdivided into: (1) Hemocompatibility, (2) Histocompatibility, and (3) Osteocompatibility. 11

**Bone marrow stromal cells** The bone marrow stroma consists of a heterogeneous population of cells that provide the structural and physiological support for giving rise to bone, cartilage, and mesenchymal cells. 17

**Cysteine** A sulfur-containing amino acid $\text{C}_3\text{H}_7\text{NO}_2\text{S}$ occurring in many proteins and glutathione and readily oxidizable to cystine, abbreviation $\text{Cys}$. 17-19, 40

**Cytotrophoblast** The inner layer of the trophoblast, interior to the syncytiotrophoblast in an embryo. It serves to anchor the embryonic chorion to the maternal endometrium. Cytotrophoblasts are stem cells in the chorionic villi. During differentiation, mononuclear cytotrophoblast fuse together into the multinucleated syncytiotrophoblasts. 21

**Density gradient** A column of liquid in which the density varies continually with position, usually as a consequence of variation of concentration of a solute. Density gradients are widely used for centrifugal and gravity induced separations of cells, organelles and macromolecules. 28

**Derivatize** Transforms a chemical compound into a product of similar chemical structure, called derivative. Generally, a specific functional group of the compound participates in the derivatization reaction and transforms the educt to a derivate of deviating reactivity, solubility, boiling point, melting point, aggregate state, or chemical composition. Resulting new chemical properties can be used for quantification or separation of the educt. 33

**Fibrous implant capsule** In newly granulation tissue, fibroblasts hypertrophy and actively synthesize collagen. With further healing there is an increasing in extracellular material, a decrease in leukocytes, macrophages, and blood vessels. The end result is a scar that surrounds the implanted prosthesis composed of collagen, fibroblasts, and blood vessels. It is a part of foreign body reaction. 10

**Fibrosis** A condition marked by increase of interstitial fibrous tissue, fibrous degeneration. 10

**Foreign body reaction** A benign variation in normal tissue response, caused by the presence of a foreign material (e.g., an implantable prosthesis). In soft tissue implantations, a “tissue capsule” composed of de novo collagen, multinucleated giant cells, fibroblasts, and blood vessels is normally seen surrounding the prosthesis. 8, 10, 13, 39, 44

**Fretting** To cause corrosion; gnaw into something, acids that fret at the strongest metals. 11

**Granuloma** A nodular inflammatory lesion that contains areas of granulation. 12

**Hapten** A small separable part of an antigen that reacts specifically with an antibody but is incapable of stimulating antibody production except in combination with an associated protein molecule. 39

**Hematopoietic** Relating to, or involved in the formation of blood cells. 8, 39
Howship’s lacuna  A groove or cavity usually containing osteoclasts that occurs in bone which is undergoing reabsorption. 22, 23

Isocratic  A procedure in which the composition of the mobile phase remains constant during the elution process. 33

Niobium  A lustrous light gray ductile metallic element that resembles tantalum chemically and is used in alloys -- symbol Nb ; called also columbium. 12

PCR  An in vitro technique for rapidly synthesizing large quantities of a given DNA segment that involves separating the DNA into its two complementary strands, binding a primer to each single strand at the end of the given DNA segment where synthesis will start, using DNA polymerase to synthesize two-stranded DNA from each single strand, and repeating the process. abbreviation PCR. 31

Podosome  The primary sites of integrin stimulated actin polymerization in leukocytes of the monocytic lineage. B cells have also been reported to form podosomes.In osteoclasts, podosomes are thought to aid in the creation of sealing rings associated with the area of bone resorption. 23

Sense and Anti-sense  describe the polarity of nucleic acid molecules. 31, 35
Sense DNA  = a single stranded DNA sequence called coding strand if the sequence is the same as that of a messenger RNA except for thymine (T) in place of uracil (U).
Anti-sense DNA  = a template strand DNA, also called the transcribed strand, which is copied to the mRNA transcript.
Anti-sense RNA  = a non-coding strand RNA complementary to the coding sequence of RNA transcript or mRNA. For example, in in situ hybridization this anti-sense RNA probe is used for detection of the RNA transcript.
Sense RNA  = not often used, mostly used in virology; in in situ hybridization the sense RNA probe is used for control hybridization.

Shear stress  The stress developed parallel to a face of a material when the layers in a cross section are gliding along each other. 11

Standard error of the mean  The standard deviation of the sample divided by the square root of the sample size:

$$SE = \frac{\hat{\sigma}}{\sqrt{n}}.$$ 33

Synovitis  Inflammation of a synovial membrane usually with pain and swelling of the joint. 39

Tribology  A branch of mechanical engineering that deals with the design, friction, wear, and lubrication of interacting surfaces (as bodily joints) in relative motion. 12

Vanadium  A grayish malleable ductile polyvalent metallic element found combined in minerals and used especially to form alloys (as vanadium steel), symbol V. 12