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Neutral proteinases and their inhibitors in the loosening of total hip prostheses

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Content

Abstract

This study demonstrated the profile of the neutral proteinases, i) matrix metalloproteinases (MMP)–1, –2, –3, and –9, and ii) serine proteinases, elastase, cathepsin G, urokinase and tissue type plasminogen activators (uPA and tPA) as well as their inhibitors, namely, tissue inhibitor of matrix metalloproteinases (TIMP)–1, α_1–antitrypsin, α_1–antichymotrypsin, plasminogen activator inhibitor (PAI)–1 & 2, around loose hip prostheses to clarify the step in the cascade of biological host response in the loosening of replaced total hip joints.

Immunohistochemical analysis showed the presence of MMPs (MMP–1, –2, –3, and –9) and serine proteinases (elastase, cathepsin G, uPA and tPA) both in the interface tissues and pseudocapsular tissues. Functional biochemical analysis revealed elevated proteolytic activities of MMPs, especially, MMP–2 and MMP–9, and also elastase and cathepsin G, which were not inhibited *in loco*, although the inhibitors, TIMP–1, α_1–antitrypsin and α_1–antichymotrypsin were detected. The results suggested the imbalance of neutral proteinase–inhibitor levels around loose hip prostheses. The proteolytic enzyme in the interface tissues could directly weaken periprosthetic tissues. The pseudocapsular tissues may induce cellular host response and proteolytic activation. Thus, the pseudocapsular tissues could contribute to the loosening via production of MMPs and serine proteinases into the synovial fluid. Pseudosynovial fluid, which showed high contents of inhibitors (TIMP–1, α_1–antitrypsin and α_1–antichymotrypsin) associated with low proteolytic potentials, could be produced to prevent the unfavorable elevation of proteolytic enzymes *in loco* as a local host response to implants.

List of orginal papers

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals.

I Takagi M, Kontinen YT, Santavirta S, Sorsa T, Eisen AZ, Nordsletten L, Suda A. Extracellular matrix metalloproteinases around loose total hip prostheses. *Acta Orthop Scand* 1994; 65 (3): 281–286.

II Takagi M, Konttinen YT, Lindy O, Sorsa T, Kurvinen H, Suda A, Santavirta S. Gelatinase / type IV collagenases in the loosening of THR endoprostheses. *Clin Orthop* 1994; 306: 136–144.

III Takagi M, Konttinen YT, Kemppinen P, Sorsa T, Tschesche H, Bläser J, Suda A, Santavirta S. Tissue inhibitor of matrix metalloproteinases–1 and collageno– and gelatinolytic potential in loose THR endoprostheses. *J Rheumatol* 1995; 22: 2285–2290.

IV Takagi M, Konttinen YT, Santavirta S, Kangaspunta P, Suda A, Rokkanen P. Cathepsin G and α_1–antichymotrypsin in the local host reaction to loosening of total hip prostheses. *J Bone Joint Surg (Am)* 1995; 77–A: 16–25.

V Takagi M, Konttinen YT, Santavirta S, Kangaspunta P, Sorsa T, Yamakawa M, Suda A. Elastase activity, uninhibited by α_1–antitrypsin, in the periprosthetic connective matrix around loose total hip prostheses. *J Orthop Res* 1995; 13: 296–304.

VI Nordsletten L, Buø L, Takagi M, Konttinen YT, Yamakawa M, Santavirta S, Aasen AO. The Plasminogen activation system is upregulated in loosening of total hip prosthesis. *Acta Orthop Scand* 1996; 67(2): 143–148.

Definitions

Biocompatibility: Affinity of artificial materials to human and animals when they are inserted.

Implant: Biomaterial placed within bone and joint space.

Interface: The junction between two different materials, or between a material and tissue.

Loosening: A process at the bone–implant interface by which prosthesis does not become osseointegrated.

Osteolysis: Destruction of bone surrounding prostheses.

Pseudocapsule: The capsular tissues surrounding artificial joint and having synovial like–membrane, which is formed after arthroplasty.

Pseudosynovial fluid: The joint fluid produced by synovial like–membrane of pseudocapsule and retained in the cavity around artificial joint.

Wear: The erosion of one or more materials that are in contact and moving relative to each other.

Proteinase: The enzyme which can cleave the bond of aminoacids and degrade the protein.

Cytokine: Chemical mediators released from various cells *in loco*.

Introduction

Progress in total hip replacement (THR) surgery has been a blessing to patients suffering from painful diseases of the hip joint, such as primary osteoarthritis, and secondary ones caused by rheumatoid arthritis, posttraumatic conditions, congenital dysplasia or dislocation and aseptic necrosis of the femoral head, whereas loosening of total hip prostheses has been one of the inevitable problems (Goldring et al. 1993, Harris et al. 1976, Maloney et al. 1990, Santavirta et al. 1990, 1993a, 1993b, 1994). Despite of intensive research, the mechanisms responsible for the loosening of total hip replacement prostheses have not been completely understood yet.

In chronic local implant reactions, the cellular response is likely to consist of monocyte/macrophages, fibroblasts, and endothelial cells (Jasty et al. 1990, Goodman et al. 1993b, Nordström et al. 1993, Santavirta et al. 1990, 1992, 1993a, 1993b, 1994). In several studies, the monocyte/macrophage-dominant area showed well-vascularized, granulomatous structure and the fibroblasts-dominant area showed dense collagenous connective tissue in the periprosthetic reactive/adaptive tissue. The local cellular reaction also typically includes scattered perivascular lymphocyte infiltrations (Santavirta et al. 1990, 1991a, 1993b, 1994). Recently several researchers have focused on immunopathological and molecular mechanisms associated with weakening of periprosthetic tissues related to biological host response to the implants (Goodman et al. 1992a, Jiranek et al. 1993, Kim et al. 1993, Santavirta et al. 1993a).

I have formulated a hypothesis on loosening of hip prostheses, which regards the loosening process as a combination of mechanical and biological processes, namely, cyclic mechanical loading combined with weakening of periprosthetic tissues as a result of biological host response, where neutral proteolytic enzymes play an important role (Figure 1).

Neutral proteinases, matrix metalloproteinases (MMPs) and serine proteinases, are considered to be among the most important proteinases related to physiological connective tissue remodeling and the subsequent turn over process. Further, these proteinases are also important for pathologic tissue destruction events such as, inflammatory diseases, including rheumatoid arthritis, cancer metastases, bone resorption, and periodontal diseases (Birkedal-Hansen 1993a, Okada et al. 1989, Sakamoto et al. 1988, Sato et al. 1993, Suomalainen et al. 1981, 1993, Woessner 1991). Especially, in pathologic tissue destructions, imbalance of proteinases and their inhibitors has been thought to cause unfavorable extracellular matrix degradation *in loco* (Krane et al. 1994, Werb 1989, Woessner 1991).

Therefore, the aim of the present study was to obtain better understanding of the biological mechanisms connected with loosening of THR prostheses, especially regarding the role of proteinases and their inhibitors. The main emphasis was on studies of the presence and role of neutral proteinases, MMPs and serine proteinases, and their inhibitors in the loosening process of total hip prostheses.

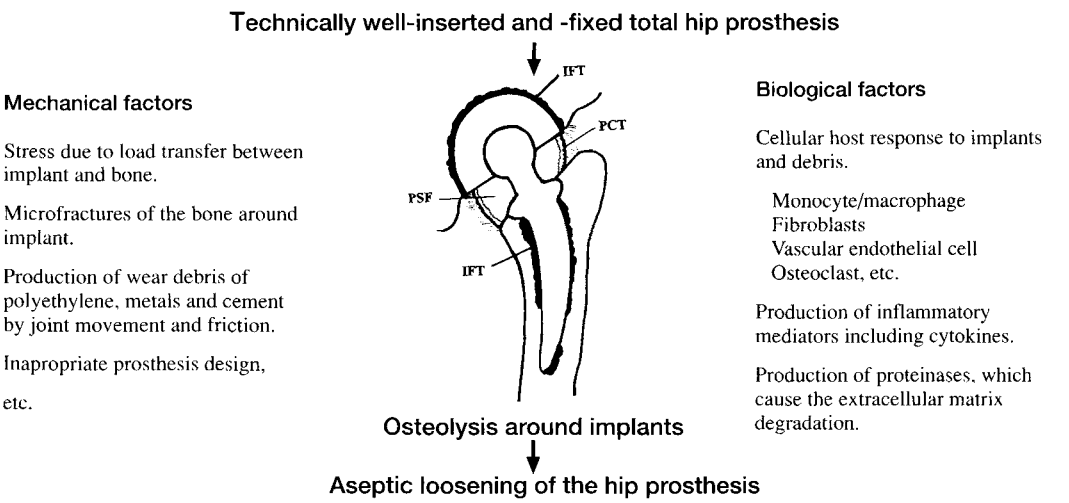

Figure 1. Hypothesis on the etiology of aseptic loosening of total hip replacement. IFT: interface tissues between bone and implant, PCT: pseudocapsular tissues formed after surgery, and PSF: pseudosynovial fluid in artificial joint space.

Review of the literature

Total hip replacement and prosthetic loosening

The clinical success of the original low–friction THR concept or related modifications is well established (Charnley 1961, 1979). These THRs have been based on methylmetacrylate fixation of the ultrahigh–molecular weight polyethylene acetabular and steel or cobalt–chromium–molybdenum (CoCrMo) alloy femoral component (Charnley 1968). However, long–term clinical results of cemented THRs, with high density polyethylene for the sockets, have shown that after 10 years the frequency of prosthesis loosening significantly increases. The adverse effects of continuos mechanical loading in the loosening process have been emphasized, and for many years it was considered that the polyethylene and methylmethacrylate wear debris would be of benign nature (Wroblewski 1979, Wroblewski et al. 1987, Editorial, Lancet 1990). When osteolysis around the cemented THR stem was first noticed, it was hypothesized that this lysis was due to a chronic low-grade infection (Charnley et al. 1968), whereas recent research has shown that massive osteolytic reactions are occasionally seen around well–fixed THR prostheses and in the definite absence of an infection (Tallroth et al. 1989, Antti–Poika et al. 1990). A great deal of controversy remains regarding the long-term reactions that occur around cemented THR prostheses and reasons which lead to implant loosening (Jasty et al. 1990). In the hope to avoid methylmethacrylate induced adverse host responses, cementless prostheses have been designed and become more common (Bjursten et al. 1990, Lalor et al. 1991, Santavirta et al. 1991a, 1991b). Also, there experiments are performed to improve the biocompatibility of THR implants (Bjursten et al. 1990, Lennox et al. 1987, Santavirta et al. 1991a, 1991b). However, the reaction to uncemented THRs which are inserted without the use of methylmethacrylate, are immunologically similar to the ones observed in cemented cases (Santavirta et al. 1991a, 1992, 1993b). Polyethylene appears to be a nearly unavoidable material in all THR designs; it is technically very difficult to find substitutes to polyethylene in the acetabular component gliding layer (Santavirta et al. 1992). Polyethylene wear debris (Revell et al. 1987) and a reaction to particulate metalic wear debris are probably the main initiators of the adverse local host reaction (Hynes et al. 1993, Maloney et al. 1993, Santavirta et al. 1991b).

In succesful THRs, where the prostheses remain well fixed over a decade, the host response has a benign character (Santavirta et al. 1991c) and signs of unfavourable host reaction may remain modest (Santavirta et al. 1992). Thus, the causes of adverse biological tissue response and osteolysis around the technically well inserted THR prostheses have still remained puzzling. Biological aspect of osteolysis or bone resorption around prostheses has been also discussed since Harris, Goldring and coworkers (Goldring et al. 1983, 1986). The presence of a synovial like–membrane between the implant and bone, periprosthetic connective tissue may impair osseointegration and may foarm a osteoclast activating factors, which can contribute to periprosthetic weakening. There is increasing concern regarding the biocompatibility of the THRs prostheses.

Histopathology in loose totally replaced hips

In revision operations for loose THR prostheses, membrane-like granulomatous tissue, referred to as "interface tissue", is found between bone and the implant. The THR is truely an artificial joint and it is surrounded by "pseudocapsular tissue", which has been in detail described in some recent papers (Kim et al. 1993, Santavirta et al. 1993b). Further, "pseudosynovial fluid" can be obtained from the artificial joint (Saari et al. 1993) and can be used for studies of the fluid phase reaction to the THR.

In "interface tissues" and "pseudocapsular tissues", as a result of chronic local reaction to the implant, the cellular response is likely to consist of monocyte/macrophages, which express CD68 and CD11b, non–specific esterase and MHC locus II coded Ia antigen, and fibroblasts, which reveal immunoreactivities to proline 4–hydroxylase and carboxy–terminal of type I procollagen (Konttinen et al. 1987, Nordström et al. 1993, Santavirta et al. 1992). In previous studies, the monocyte/macrophage–dominant area showed well–vascularized, granulomatous structure and the fibroblast–dominant area showed dense collagenous connective tissue. The local celluller reaction also commonly includes scattered perivascular T lymphocyte infiltrations mostly consisting of the CD4 positive T lymphocyte subset (Santavirta et al. 1990, 1994).

Thus, implants cause a complex reactive tissue and cell response (Gristina et al. 1987, Hynes et al. 1993, Davis et al. 1993, Jiranek et al. 1993, Maloney et al.

1993, Santavirta et al. 1990, 1993b, 1994). The implant and its wear products represent a large foreign body surface, which in some way have to be integrated into the organic tissue matrix. Monocyte/macrophages are able to phagocytose small foreign particles (Gristina et al. 1987), but not to digest such particles. This may lead to exaggerated local host response. New cells are recruited by cytokines secreted by the initially stimulated monocyte/macrophages (Horowitz et al. 1993, Davis et al. 1993, Goodman et al. 1993b, Jiranek et al. 1993, Wahl et al. 1988). Further, implant material may act as a source of electrons. Titanium, like iron and copper, is able to donate or accept electrons. Redox reactions may lead to formation of reactive molecules (superoxide, hydrogen, radicals, organic radicals) with tissue destructive potential (Saari et al. 1992). Although monocyte/macrophages, acessory antigen presenting cells and immunocompetent lymphocytes are important participants in the local tissue /cell response, connective tissue cells are also important in the reactive process. Resident osteoblasts, osteoclasts and fibroblasts synthesize and exert considerable control of the extracellular matrix. It is this matrix and its quality, which is responsible for the loosening or good–lasting fixation of the implants. Especially, quality of bone is important to support the inserted implants. It has been strongly suggested that wear particle–induced release of inflammatory mediators, cytokines, can play an important role in the osteolysis around prosthesis (Goldring et al. 1983, Howie et al. 1993, Jiranek et al. 1993, Horowiz et al 1993). Osteoclasts are activated by inflammatory mediators, such as interleukin (IL)–1, IL–6, tumor necrosis factor (TNF)–α, platelet–derived growth factor (PDGF), and prostaglandin $(PGE)_2$, and able to induce bone resorption (Hynes et al. 1993, Santavirta et al. 1994). However, the whole cascade and mechanism of foreign body reaction, periprosthetic weakening and bone resorption around prostheses has not been clarified yet.

Proteolytic enzymes in connective tissues

There are four types of proteinases that can be classified by their catalytic mechanisms (Table 1). Aspartic and cysteine proteinases are mostly active at acidic pH, but serine proteinases and MMPs are active at neutral and slightly alkaline pH (Werb 1989). Matrix degradation in vivo is mainly achieved in extracellular space, which is maintained at neutral pH, except in the pericellular space of inflammatory cells. Therefore, neutral proteinases, MMPs and serine proteinases, may play an important role in matrix degradation.

Table 1. Proteinases of connective tissues (Werb 1989)

Class	Enzymes	Optimal pH
Aspartic	Cathepsin D	3–6
Cysteine	Cathepsin B Cathepsin L Cathepsin N	3–7
Serine	PMN elastase Cathepsin G Plasminogen Activators (Tissue type) (Urokinase type) Plasmin Plasma kallikrein Mast cell chymase I, II Tryptase	6–10
Metallo	Matrix metalloproteinases	6–9

MMPs are a family of at least 12 homologous enzymes, which are able to degrade almost all components of the extracellular matrix (Table 2). MMPs take part in the extracellular matrix degradation in both pysiological tissue remodelling and pathological conditions, such as wound healing, cancer and growth of metastases, rheumatoid arthritis, periodontal diseases (Birkedal–Hansen 1993a, 1993b, Birkedal–Hansen et al. 1988, Freije et al. 1996, Knäuper et al 1996, Saari 1992, Sato et al. 1993, 1994, Takino et al. 1996, Woessner 1991).

MMPs share similarities within their structure. The largest structure among MMPs, 92 kD gelatinase or MMP–9 has seven domains. All other MMPs which have been so far structurally characterized, lack one or more of these domains. The seven domains are: 1) a 17–29 residue signal peptide, 2) a 77–87 residue propeptide domain, which constitutes of the NH_2–terminal domain of the secreted pro–MMPs, 3) a domain of the active–enzyme that is separated from the putative 4) zinc–binding site by 5) a fibronectin–like domain. 6) a type V collagen–like domain and 7) a COOH–terminal hemopexin–like domain. The proenzyme domain contains the cystein, which most likely binds to Zn^{2+} on zinc–binding site. It is believed to play a role in maintenance of catalytic latency of the precursor form of MMPs. The fibronectin like domain is found only in MMP–2 and MMP–9, and is considered to facilitate binding of the two gelatinases to gelatins. The role of type V collagen–like domain, which is found only in MMP–9, is unknown. The hemopexin–like domain, which is absent from matriysin or MMP–7, appears to play a role in encoding substrate specifisity (Birkedal–Hansen et al. 1993, Wilhelm et al. 1989, Woessner 1991). Recently,

Table 2. Matrix metalloproteinases (MMPs)

Enzyme	MMP No. EC No.	Mollecular weight (kD) Proenzyme / Active form	Substrate	Reference
I. Interstitial collagenase group				
Fibroblast type	MMP–1 3.4.24.7	52000 / 41000	I, II, III, VII, X collagen gelatin,	Goldberg et al. 1986
Neutrophil type	MMP–8 3.4.24.34	58000 / 57000	I, II, III collagen	Hasty et al. 1990
Collagenase–3	MMP–13	65000 / 55000	I collagen	Knäuper et al. 1996
II. Gelatinase/IV collagenase group				
Gelatinase A (72 kD gelatinase)	MMP–2 3.4.24.24	72000 / 67000	IV, V, VII, X collagen gelatin, fibronectin	Collier et al. 1988
Gelatinase B (92 kD gelatinase)	MMP–9 3.4.24.35	92000 / 67000	IV, V, VII, X collagen gelatin	Goldberg et al. 1989
III. Stromelysin group				
Stromelysin–1	MMP–3 3.4.24.17	57000 / 45000 28000	proteoglycan, fibronectin, IV, VII, IX collagen, gelatin, laminin	Wilhelm et al. 1987
Stromelysin–2	MMP–10 3.4.24.22	57000 / 48000 28000	same as MMP–3	Müller et al. 1988
IV. Others				
Matrilysin (PUMP–1) (Uterine metalloproteinase)	MMP–7 3.4.24.23	28000 / 19000	gelatin, fibronectin, proteoglycan, laminin	Müller et al. 1988
Stromelysin–3	MMP–11	58000 / 28000	—	Basset et al. 1990
Macrophage–Metalloelastase	MMP–12	54000 / 45000 22000	elastin	Shapiro et al. 1992
Membrane type MMPs	MT–MMPs	—	—	Sato et al. 1994 Takino et al. 1995

MMP No.: Matrix metalloproteinase number, EC No. : Extracellular matrix number.

membrane–type MMPs has been reported. They have transmembrane and intracytoplasmic domains (Sato et al. 1993, 1994, Takino et al. 1995).

In most cell types, MMPs are not stored, but their synthesis and secretion is regulated mainly by transcription of MMP genes by several cytokines and growth factors. Generally these cells, fibroblasts, keratinocytes, monocyte/macrophages, and endothelial cells respond to several cytokines and growth factors, such as IL–1, TNF–α, Transforming growth factor (TGF)–α, epidermal growth factor (EGF), PDGF (Birkedal–Hansen 1993b). However, among these responses there are important cell–specific and cytokine–specific differences (Birkedal–Hansen 1993a, 1993b). In fibroblast, IL–1α induces primarily stromelysin–1 (MMP–3), TNF–α primarily MMP–1 (Dayer et al. 1985, MacNaul et al. 1990). TGF–α down regulates MMP–1 and MMP–3, but up–regulates MMP–2 in fibroblasts, whereas in keratinocytes, TGF–α up–regulates both MMP–2 and MMP–9 (Kerr et al. 1990, Salo et al. 1991). IL–1α, which is the most potent known inducer of MMP–1 and MMP–3 expression by fibroblasts, has no effect, at least in humans, on keratinocytes (Birkedal–Hansen 1993a, Birkedal–Hansen et al. 1993, Frisch et al. 1987). However, polymorphonuclear neutrophil (PMN) type MMPs (MMP–8 and MMP–9) are already synthesized during the developement of PMNs in bone marrow. They are stored in intracellular granules and upon different and granule–specific stimuli degranulated to the extracellular space in a process involving membrane fusion and exocytosis (Bainton et al. 1971, Birkedal–Hansen et al. 1993, Woessner 1991). MMPs are secreted in latent, zymogen form (Weiss 1989) and can be activated by proteolytic and non–proteolytic means *in vitro*, but the *in vivo* activation mechanisms have not been completely clarified yet (Knäuper et al. 1993, Lindy et al. 1986, Nagase et al. 1990, Saari 1992, Saari et al. 1990, Weiss 1989).

MMPs are dependent on Zn^{2+} ions for activity, and they are additionally stabilized and activated by Ca^{2+}. Interstitial collagenase group, MMP–1 (Goldberg et al. 1986) and MMP–8 (Hasty et al. 1990), are major factors associated with the degradation of collagenous extracellular matrices, which are mainly type I and III collagens. They cleave the three α–chains of the triple helix of the interstitial collagens, leading to the formation of the characteristic three–fourth to one–fourth length degradation products (Sorsa et al. 1992). These fragments spontaneously lose their heli-

cal structure and are denatured into gelatin at body temperature. The gelatin is digested by gelatinases / type IV collagenases group, namely, MMP–2 (Collier et al. 1988) and MMP–9 (Wilhelm et al. 1989), which also degrade type IV collagen, which is one of the components of the basement membrane. In the stromelysin group, MMP–3 (Wilhelm et al. 1987) degrades proteoglycan, type III, IV, VII collagens, fibronectins and gelatin, and MMP–10 (Müller et al. 1988) degrades type III, IV, and V collagens, fibronectin and gelatin. In addition, recently, new type of interstitial collagenase (collagenase 3, MMP–13, Knäuper et al. 1996, Freije et al. 1996), MMP–7 (Müller et al. 1988), MMP–11 (Basset et al. 1990) have been reported. Further, membrane type MMPs, which are considered to be deeply related to tumor invasion (Sato et al. 1994, Takino et al. 1995) have been reported and form still another group in the MMP gene family.

Inhibitors of matrix metalloproteinases

Plasma and fluid levels of MMP activities are mainly controlled by α_2–macroglbulin (α_2M), whereas, MMPs activities of tissue levels or intracellular levels are controlled by their endogenous inhibitor, Tissue inhibitor of matrix metalloproteinase (TIMP)–1. It is a mannose–rich sialoglycoprotein of Mr 28K (Burnning et al. 1984, Carmichael et al. 1986, Stricklin et al. 1983b), which forms 1:1 noncovalent complexes with active MMPs, with a Ki of 10^{-10} M for collagenase (Cawston et al. 1983). Other members of the TIMP family, TIMP–2, a two chain form of TIMP–2 (tc–TIMP), TIMP–3 and TIMP–like large inhibitor of matrix metalloproteinase (LIMP) have been recognized (Abe et al. 1972, Clark et al. 1993, De Clerck et al. 1989, Docherty et al. 1985, Cawson et al. 1990, Goldberg et al. 1989, Krane et al. 1994, Leco et al. 1994, Miyazaki et al. 1993, Pavloff et al. 1992, Stetler–Stevenson et al. 1989).

Serine proteinase

Serine proteinases have a catalytically essential serine residue at their active site, are most active at about neutral pH, and form the largest class of mammalian proteinases. Serine proteinases participate in the cascade of coagulation, fibrynolysis and complement activation, like plasmin, thrombin, and plasma kallikrein. PMN elastase, cathepsin G, plasminogen activators (tissue type and urokinase type), plasmin, plasma kallikrein, mast cell chymase I and II, and tryptase are considered to take part in extracellular matrix degradation (Okada et al. 1988, Werb 1989) (Table 1). In addition, elastase and cathepsin G are well known to be involved in arthritis and joint destruction (Janusz et al. 1991).

Elastase (28 kD) (Shinha et al. 1987) is a member of serine proteinase enzyme group, which enzymes have a catalytically essential serine residue at their active site and, in contrast to asparatic acid and cysteine proteinases with acidic pH optima, are most active at neutral pH. Therefore, elastase is active against matrix components upon release to the extracellular space from the cells. Elastase is produced and/or stored by PMNs and monocyte/macrophages (Barrett 1980, Campbell et al. 1989, Werb 1989, Werb et al. 1975). In contrast to PMNs, in which elastase is stored in the azurophilic granules until the cell is triggered to exocytosis (Bentwood et al. 1980, Damiano et al. 1988, Dewald et al. 1975), activated monocyte/macrophages continuously synthesize and secrete elastase (Werb et al. 1975). Furthermore, matrix metalloproteinases are secreted as latent proenzymes (Murphy et al. 1985, Nagase et al. 1981), whereas elastase is secreted as an active enzyme species. Elastase can degrade elastin (Boudier et al. 1991), laminin (Heck et al. 1990), type IV collagen (Pipoly et al. 1987), proteoglycans (Janusz et al. 1991) and fibronectin (McDonald et al. 1980). Furthermore, it has been demonstrated that elastase activates matrix metalloproteinase–3 proenzyme (Nagase et al. 1990), which, as described above, is also an important proteolytic enzyme. Elastase decreases the activity of tissue inhibitor of metalloproteinases (Okada et al. 1988), which is an endogenous inhibitor of MMPs, and elastase modulates TNF–α (Scuderi et al. 1991), which is a cytokine able to induce collagenase production (Dayer et al. 1985). Interestingly, MMPs inactivate α_1–antitrypsin (Desrochers et al. 1988, Vissers et al. 1988), which can inhibit elastase activity (Abbink et al 1993, Beatty et al. 1980, Travis 1988). These *in vitro* results suggest that elastase may play a critical role in matrix degradation, not only as a proteolytic enzyme able to degrade extracellular matrix, but also by modulating other such enzymes. However, contribution of elastase–α_1–antitrypsin balance to activated proteolytic cascade in loose hip prostheses *in situ* has not been known yet.

Cathepsin G (28–30 kD) is also a member of serine proteinases as well as elastase and is most active at neutral pH. Therefore, cathepsin G is active against matrix components upon release into the extracellular space from azurophilic granules of the PMNs and monocytes (Boudier et al. 1991, Heck et al 1990, Janusz et al. 1991, Piploy et al. 1987, Travis 1988,

Werb 1989). Furthermore, it has been demonstrated that cathepsin G activates MMPs (Chatham et al. 1992), which, as described above, are also important proteolytic enzymes. Cathepsin G can decrease the activity of TIMPs (Okada et al. 1988), which is an inhibitor of MMPs, and modulates TNF–α (Renesto et al. 1991), which is a cytokine able to induce collagenase production (Dayer et al. 1985). These *in vitro* results suggest that cathepsin G may play a role in matrix degradation, not only as a proteolytic enzyme able to degrade extracellular matrix, but also as a modulator of other such enzymes.

There are two distinct genes for plasminogen activators, namely, urokinase type plasminogen activators (uPA) and tissue type plasminogen activators (tPA). uPA is a trypsin–like serine proteinase secreted as a proenzyme of 55kDa, which must be cleaved into two chains of 20 kDa and 34 kDa connected by a single disulfide bridge for activity (Collen 1987). Plasmin is able to perform this activation cleavage. tPA is a serine proteinase of 72 kDa, which is activated by removal of an activation peptide. It can be converted to a two–chain form by plasmin. Plasminogen activators are secreted proteinases that are produced by macrophages (Blasi et al. 1987, Vasalli et al. 1984), fibroblasts(Werb and Aggeler 1978,), synovial cells (Berger et al. 1977, Hamilton et al. 1981), endothelial cells(Sakela 1985), and polymorphonuclear leukocyte (Sakela 1985). Both proenzyme and active form of uPA bind tightly to the surface of macrophages and fibroblast via a specific saturable receptor, which can folcalize the enzyme activity to pericellular space, and protect the enzyme from endogenous inhibitors (Werb 1989).

Inhibitors of serine proteinases

The activities of serine proteinases *in vivo* are modulated by the naturally occurring inhibitors. In addition to α_2M, the inhibitors also include "serpins", which are the most prominent and spesific inhibitors of serine proteinases, and are glycoproteins of 50–100 kD (Stryer 1988), such as α_1–antitrypsin (α_1–proteinase inhibitor), α_1–antichymotrypsin, antithrombin III, C1–inhibitor, α_2–antiplasmin, protease nexin–1, 2 and 3, and plasminogen activator inhibitor (PAI)–1 and PAI–2. Their physiologic importance is reflected by the fact that serine proteinase inhibitors form about 10 percent of all plasma protein.

α_1–antitrypsin is a glycoprotein (51–52 kD) which exists in a number of genetic varients. It is mainly synthesized in the liver, but also synthesized and secreted locally in tissues by monocyte/macrophages (Koj et al. 1978). It is composed of a single polypeptide chian of 394 methionine at position 358 (Mega et al. 1980, Carrell et al. 1982). α_1–antitrypsin is the main inhibitor of elastase, which is also regulated by α_2M and secretory leukoproteinase inhibitor (Weiss, 1989).

α_1–antichymotrypsin is a glycoprotein (MW 69 kD) which main biological function is the inhibition of activated phagocytes. It acts as an acute phase reactants and it may increase up to 3–4 times in condition with sever inflammatory change. α_1–antichymotrypsin is the main inhibitor of cathepsin G, which is also regulated by α_2M and the secretory leukoproteinase inhibitor (Weiss, 1989).

uPA is inhibited by protease nexin–1, an inhibitor produced by fibroblasts (Eaton and Baker 1983) and PAI–1 and 2, produced by fibroblasts, macrophages, and endothelial cells (Blasi et al. 1986, 1987, Medcalf et al. 1988). tPA is inhibited by PAI–1 and –2 (Blasi et al. 1987, Medcalf et al. 1987).

Aim and outlines of the study

Aim of the study

Despite of intensive research, the molecular mechanisms of the local tissue reaction that leads to adverse biological response and osteolysis around the technically well inserted THR prostheses have remained unclear. There is increasing concern regarding the biocompatibility of THR implants and cellular host response associated with loosening of hip prostheses is currently an important topic in orthopaedic research. In this context, proteolytic enzymes are considered to play an important role in the weakening of the periprosthetic connective tissues.

Extracellular matrix degradation *in vivo* occurs in the extracellular space at neutral pH. Therefore, neutral proteinases, which are active at physiological pH, are considered important in the cascade of connective tissue degradation in both normal tissues and pathological conditions. Thus, MMPs and serine proteinases are thought to be the most important enzymes which are responsible for extracellular matrix degradation around THR prostheses.

Therefore, the aim of the present study was to obtain better understanding of biological mechanisms associated with loosening of THR prostheses at the proteinase–inhibitor level. Thus, the present study focuses on investigation of the presence and role of neutral proteinases, MMPs and serine proteinases, and their inhibitors in the loosening of THR prostheses.

Outlines of the study

I. To study immunohistochemically the presence of neutral proteinases and their inhibitors in the local loosening reaction of THR prostheses; interstitial collagenase (MMP–1 and MMP–8), gelatinase/type IV collagenase (MMP–2 and MMP–9), stromelysin–1 (MMP–3), TIMP–1, PMN type elastase, cathepsin G, uPA, tPA, α_1–antitrypsin, α_1–antichymotrypsin, PAI–1, and PAI–2.

II. To investigate collageno– and gelatinolytic activity uninhibited by endogenous inhibitors in the periprosthetic tissues around loose THR prostheses by using DNP–S degradation test with reverse phase HPLC system.

III. To examine gelatinase/type IV collagenase activity around loose THR prostheses by using a zymographic method.

IV. To measure TIMP–1 content around THR loose prostheses by enzyme–linked immunosorbent assay (ELISA).

V. To evaluate elastase and cathepsin G activities around THR prostheses by spectrophotometry with synthetic substrates.

VI. To measure α_1–antitrypsin and α_1–antichymotrypsin contents around loose THR prostheses by laser nephlometric assay.

VII. To identify the presence and molecular form of elastase, cathepsin G, α_1–antitrypsin and α_1–antichymotrypsin around loose hip prostheses by immunoblot analysis.

VIII. To measure the levels of tPA, uPA, PAI–1, and PAI–2 by ELISA.

Material and methods

Patients and diagnoses

Patients with loosening of totally replaced hips

In total, 17 patients who had revision THR for aseptic loosening of totally replaced hips were included in this study. Nine of the 17 patients were women and 8 were men, and their mean age was 65 (46–81) years. Thirteen THRs were originally performed due to primary osteoarthritis, and four to treat secondary osteoarthritis caused by congenital dislocation of the hip joint. Type of alloy of the THRs to be revised was Co-Cr-Mo in 11 cases and Ti-Al-V(titanium-aluminum-vanadium alloy) in 6 cases. Type of fixation was "cemented" in 6 cases and "cementless" in 11 cases (I–VI).

Control samples

Capsular tissues of hip joints were obtained from six patients with medial type of femoral neck fracture. Five of these hip fracture patients were women and one was a man. Their mean age was 76 (63–85) years and mean time from accident to operation was 19.3 (8–24) hours (I). Further, synovial samples were obtained from 8 patients who had knee arthroscopy to examine meniscus injury. The knees were clinically not inflammatory and the patients did not have osteoarthritic changes or traumatic hemoarthrosis. Four of the 8 patients were women and four were men, and their mean age was 34 (20–46) years. Six of the patients had medial meniscus lesions and two did not (II–VI).

Reagents

The reagents used for cell biologic, biochemical, immunochemical and immunohistochemical techniques in this study were of the highest analytical reagent grade (I–VI).

Sample collection, processing and storage

In THR revision operations performed for loosening of prostheses, samples of the interface tissues (I–VI), of the pseudocapsular tissues (I–VI) and pseudosynovial fluid (III, IV, V) were collected. In operations to treat acute femoral neck fractures (I) and knee arthroscopies on clinically noninflammatory knees (II–VI), synovial capsular tissues were collected as controls. These samples were embedded and frozen in OCT compound and kept until immunohistochemical analysis (IV–VI). The samples were also kept until biochemical assay (II–VI).

Immunohistochemistry

Cryostat sections were used for immunohistochemistry. Avidin–biotin–peroxidase complex method (Hsu et al. 1981, I, III–V) and alkaline phosphatase anti alkaline phosphatase technique (Buø et al. 1993, VI) were applied.

Biochemical assay

Tissue extraction and dialysis

Tissue samples were homogenized in ice bath after addition of neutral salt extraction buffer (II–VI). Fluid samples were also mixed with the same volume of the neutral salt extraction buffer (III–V). After incubation and centrifugation, the samples were dialyzed for MMP assay (II, III, VI), and for serine proteinase assay (IV, V).

Assay for collagenolytic and gelatinolytic activities

DNP–S (III) sensitive collagenolytic and gelatinolytic activity, uninhibited by endogenous inhibitors, was measured with synthetic substrate and high performance liquid chromatography (HPLC; Gray et al. 1982, Masui et al. 1977, Seltzer et al. 1987). In the presense of the enzymes the DNP–S substrate was separated from the two product peptides, DNP–P (III) and Ile–Ala–Gly–Gln–D–Arg at ambient temperature using HPLC system C_{18} reverse–phase colum. Degradation rate of DNP–S substrate was expressed as (DNP–P/DNP– S) x 100 (%)(III).

Zymographic and densitometric analysis

IL–1α stimulated–fibroblast culture supernatant was uased as MMP–2 control. Human blood leukocytes were prepared (Konttinen et al. 1991, Stricklin et al. 1983a) and the gelatinase containing fractions of PMN were stored as MMP–9 control. The tissue extracts of interface, pseudocapsular and control tissue samples, fibroblast supernatant (MMP–2), and PMN extract (MMP–9) samples were analyzed with zymographic technique (II). The degradation activity of ge-

latinase/type IV collagenase was verified densitometrically. The percent (%) area degradation was calculated and relative activities of MMP–2 and MMP–9 were expressed as % area degradation of each sample / % area degradation of MMP–2 or MMP–9 control. Type IV collagen was also used to examine the substrate specificity of the enzymes in the samples (II).

Spectrophotometric enzyme assay

The measurement of cathepsin G activity was performed by SAAPPNA (IV) as a substrate, and that of elastase was by SAAVNA (V), respectively (Nakajima et al. 1979). Cathepsin G and elastas values (IU/L) were obtained from calibration curves based on a plot of initial velocities ($V_o = \Delta OD_{405nm} / \Delta t_{20-50\ min.}$) of cathepsin G and elastase standards, respectively (IV, V).

Laser nephelometric enzyme assay

Laser nephelometric immunoassay was performed to measure α_1–antitrypsin and α_1–antichymotrypsin content (Tuenguler et al. 1988). Tissue extract samples and pseudosynovial fluid samples were diluted and mixed with polyclonal anti–human α_1–antichymotrypsin or α_1–antitrypsin and reaction buffer (pH 7.2). The light scattering produced by antigen–antibody complex reaction was measured (III, IV).

Enzyme–linked immunosorbent assay

Competitive ELISA was performed according to Günther et al. (1994) (III). Antigen level analysis of uPA, tPA, PAI–1 and PAI–2 was performed by ELISA kit system (VI).

Gel electrophoresis and immunoblot analysis

Sodium dodecyle sulphate / polyacrylamide gel–electrophoresis (SDS/PAGE) with 10% polyacrylamide gel was performed according to a modified method of Laemmli (1980). After electrophoresis the gels were blotted onto nitrocellulose membrane as described by Towbin et al. (1974). After incubation with the antibodies, alkaline phosphatase technique was applied for the detection of the enzymes (III, IV).

Statistics

Results were expressed as mean ± standard error of mean. Statistical values and their significances were determined using analysis of variance with Scheffe *F*–test (I, IV) or with Fisher's propability by least significant difference post hoc test (II), unpaired Student *t*–test (III), Kruskal–Wallis test followed by Mann–Whitney's U test (V). Mann–Whitney U and Wilcoxon signed rank test (VI).

Results

Matrix metalloproteinases

Collagenolytic and gelatinolytic activity in loose totally replaced hips

DNP-S sensitive collagenolytic and gelatinolytic activity was significantly higher in the interface tissue samples than in the pseudocapsular tissue samples (p=0.01) and pseudosynovial fluid samples (p=0.002), and was also significantly higher in the pseudocapsular tissues than in the pseudosynovial fluid samples (p=0.002) (V, Table 3).

Interstitial collagenase (MMP-1 and MMP-8)

MMP-1 was observed in both interface and pseudocapsular tissues in loose THRs. Monocyte/macrophages, fibroblasts and vascular endothelial cells revealed marked intracellular immunoreactivity to MMP-1. In synovial capsular tissues obtained from patients with fractured hips, the synovial lining cell layer revealed slight to moderate MMP-1 immunoreactivity. Only occasional stromal fibroblasts and vascular endothelial cells showed slight positive immunostainig for MMP-1. The number of MMP-1 positive cells in the interface tissues and in the pseudocapsular tissues in loose THRs were significantly higher than those found in the synovial capsular tissues of hips with femoral neck fractures (p=0.001) (I, Table 3).

Immunoreactivity to MMP-8 was only observed in scattered polymorphonuclear leukocytes in both interface and pseudocapsular tissues from loose THRs. In contrast, marked MMP-8 immunoreactivity was observed in PMNs associated with or close to the synovial membrane and in or around the vessels in the subsynovial connective tissues of fractured hips (I, Table 3).

Gelatinase/type IV collagenase (MMP-2 and MMP-9)

In both the interface and pseudocapsular tissues, fibroblasts stained strongly positive for MMP-2 whereas monocyte/macrophages showed moderate immunostaining for MMP-2. Some of the synovial lining cells and stromal fibroblasts of fractured hip samples showed slight MMP-2 immunoreactivity. The number of MMP-2 positive cells in the interface tissues and in the pseudocapsular tissues was significantly higher than that in the synovial capsular tissues of fractured hips (p=0.0002) (I, Table 3).

In both the interface and pseudocapsular tissues around loose prostheses, monocyte/macrophages revealed marked intracytoplasmic MMP-9 immunoreactivitiy, and fibroblasts and vascular endothelial cells of these tissues showed moderate immunostaining for MMP-9. MMP-9 positive PMNs were rarely observed. In the synovial capsular tissues of fractured hip, slight MMP-9 immunostaining was observed in the synovial lining cell layer, stromal fibroblasts and PMNs. In the interface tissue samples, the number of MMP-9 positive cells in the interface and pseudocapsular tissues in loose prostheses was much higher than that in the synovial/capsular tissues of fractured hips (p=0.0001) (I, Table 3).

Zymographic analysis revealed gelatinolytic bands corresponding to MMP-2 control (fibroblast supernatant, 72 kDa) and MMP-9 control (PMN extract, 92 kDa) both in the interface tissue and pseudocapsular tissue samples. In the control tissue samples, a gelatinolytic band corresponding to MMP-2 control was observed, but MMP-9 was not found in any of the control samples. In accordance with the result of zymography, densitometric analysis revealed gelatinolytic peaks corresponding to MMP-2 control (fibroblast supernatant, 72 kDa) and MMP-9 control (PMN extract, 92 kDa) both in the interface tissue and pseudocapsular tissue samples. In the control samples, a gelatinolytic peak corresponding to MMP-2 but not to MMP-9 control was detected.

Relative gelatinolytic activity of MMP-2 was higher in the interface tissue samples and pseudocapsular tissue samples than in the control tissue samples (p=0.001). Relative gelatinolytic activity of MMP-9 was higher in the interface tissue samples than in the pseudocapsular tissue samples (p=0.001). MMP-9 type gelatinolytic activity was absent in the control samples (II, Table 3).

Type IV collagenolytic potential was found in all samples in accordance with the results of gelatinolytic potential (II, Table 3).

Stromelysin-1 (MMP-3)

Monocyte/macrophages, fibroblasts and vascular endothelial cells in both interface and pseudocapsular tissues in loose endoprostheses revealed moderate to markedly positive immunoreactivity to MMP-3. In the samples from fractured hips, staining of synovial lining cells, stromal fibroblasts, and vascular endothelial cells was weaker. The number of MMP-3 positive cells in the interface and pseudocapsular tissues in loose THRs were significantly higher than recorded in the synovial/capsular tissues obtained from fractured hips (p=0.0001) (I, Table 3).

Table 3. Immunohistochemical and biochemical analysis of the samples

	Interface	Pseudo-capsule	Pseudo-synovial fluid	Control tissues
Collageno- and gelatinolytic activity (%)	56 ± 6	32 ± 4	6 ± 2	—
MMP-1 immunoreactive cell (cells / HPF)	218 ± 20	196 ± 16	—	54 ± 13
MMP-8 immunoreactive cell (cells / HPF)	5 ± 2	4 ± 1	—	9 ± 2
MMP-2 immunoreactive cell (cells / HPF)	114 ± 12	133 ± 24	—	17 ± 4
MMP-9 immunoreactive cell (cells / HPF)	180 ± 12	164 ± 9	—	22 ± 6
MMP-3 immunoreactive cell (cells / HPF)	184 ± 20	172 ± 17	—	45 ± 7
MMP-2 activity	10.4 ± 1.0	9.8 ± 0.9	—	2.5 ± 0.4
MMP-9 activity	6.4 ± 0.5	2.5 ± 0.4	—	0 ± 0
TIMP-1 (ng / ml)	795 ± 203	2026 ± 584	1267 ± 494	—
Elastase immunoreactive cells (cells / HPF)	21.7 ± 2.3	19.8 ± 2.2	—	1.6 ± 0.3
Elastase activity (IU / ml)	70.9 ± 9.9	42.9 ± 7.4	—	0.1 ± 0.1
Cathepsin G activity (IU / ml)	31.9 ± 5.7	15.5 ± 4.2	2.5 ± 0.5	2.4 ± 0.9
uPA (ng / ml)	18.1 ± 1.8	15.3 ± 2.2	—	4.6 ± 0.8
tPA (ng / ml)	22.2 ± 3.9	28.7 ± 10.6	—	76.0 ± 9.7
α_1-antitrypsin immunoreactive cells (cells / HPF)	0.2 ± 0.1	0.4 ± 0.2	—	0.2 ± 0.1
α_1-antitrypsin (µg / ml)	25.3 ± 5.7	11.2 ± 2.0	—	11.0 ± 2.2
α_1-antichymotrypsin (µg / ml)	35.9 ± 6.4	25.5 ± 3.3	185.7 ± 46.4	16.6 ± 5.7
PAI-1 (ng / ml)	77.5 ± 14.4	81.1 ± 16.8	—	4.3 ± 2.1
PAI-2 (ng / ml)	4.4 ± 1.3	4.7 ± 1.1	—	2.3 ± 1.8

Tissue inhibitor of metalloproteinases

Tissue inhibitor of metalloproteinases (TIMP)-1

Moderate to marked immunoreactivity of TIMP-1 was observed in pseudosynovial lining cells both in the interface and pseudocapsular tissues around loose THR endoprostheses. Weak to moderate reactivity was observed in monocyte/macrophages, endothelial cells and fibroblasts, both in the interface and pseudocapsular tissues in loose THRs (V).

TIMP-1 level was low in the interface tissues between bone and implants compared to the pseudosynovial fluid samples, but not to the pseudocapsular tissue samples from loose THRs (p=0.2; V, Table 3).

Serine proteinases

Elastase

Immunoreactivity to elastase was observed in the interface tissue and the pseudocapsular tissue samples obtained from revisions of loose hip prostheses. Interstitial monocyte/macrophage-like mononuclear cells and occasional PMNs in the interface tissue and the pseudocapsular tissue samples showed intense intracytoplasmic immunostaining. The number of elastase-immunoreactive cells was higher both in the interface tissue samples and the pseudocapsular tissue samples than was the case in the control synovial tissue samples (p=0.001) (IV, Table 3).

Elevated elastase activity was higher in the interface tissue samples and the pseudocapsular tissue samples of loose THRs when compared to that found in the control synovial tissue samples (p=0.001). The pseudosynovial fluid samples from loose THRs showed low elastase activity (IV, Table 3).

Elastase-immunoreactive bands, corresponding to positive control in the 26–28 kDa area, were observed moderately in the interface tissue samples and in the pseudocapsular tissue samples, but the bands were more weak in the control synovial tissue samples. Although the activity of elastase in the pseudosynovial fluid samples was low, there was a prominent elastase-immunoreactive band to be seen (IV, Table 3).

Cathepsin G

Immunoreactivity to cathepsin G was observed in the interface tissue and the pseudocapsular tissue samples which were obtained at revisions in the loosening of THR endoprostheses. Monocyte/macrophages and fibroblasts of the interface tissue and pseudocapsular tissue samples showed moderate intracytoplasmic immunostaining in highly cellular area, whereas the few scattered polymorphonuclear neutrophils in or around blood vessels showed intense immunostainig, and pseudosynovial lining cells were weakly or moderately positive. In the control capsular samples, synovial lining cells were weakly positive or negative. As highly cellular area was greater in the interface tissues than in the pseudocapsular tissues, cathe-

psin G–immunoreactive cells were abundant in the interface tissues when compared to the pseudocapsular tissues (III, Table 3).

Cathepsin G activity was detected in the interface tissue samples and the pseudocapsular tissue samples ($p<0.01$). Cathepsin G activity of the control capsular tissue samples was lower than that of the periprosthetic interface samples and pseudocapsular tissue samples ($p<0.01$). The THR pseudosynovial fluid samples also showed low cathepsin G activitiy ($p<0.01$), which was significantly lower in comparison to other periprosthetic samples (III, Table 3).

Immunoblot analysis showed immunoreactive cathepsin G, corresponding to positive control in the 28–30 kDa area, not only in the interface tissue samples and the pseudocapsular tissue samples, but also in the THR pseudosynovial fluid samples. Additional marked immunoreactive bands of the tissue samples and the THR pseudosynovial fluid were also observed in 60–70 kDa and 70–80 kDa areas. Although the activity of cathepsin G in the pseudosynovial fluid samples was low, the immunoreactive band was intense. Immunoreactive band of control capsular tissue samples corresponding to cathepsin G control was weak (III, Table 3).

Plasminogen activators (urokinase type and tissue type)

Interface tissue samples and pseudocapsular tissue samples were positive for uPA and tPA. Most cells staining for uPA or tPA were heavily loaded with metals, polyethylene or cement particles, which indicated that uPA and tPA positive cells were monocytes/macrophages.

The concentration of uPA in the interface tissue samples and in the pseudocapsular tissue samples was significantly higher than in the control capsular tissue samples ($p=0.0001$ for interface tissues and $p=0.0003$ for pseudocapsular tissues). tPA level was lower in the interface tissue samples ($p=0.0002$) and pseudocapsular tissue samples ($p=0.0005$), when compared to that in the control capsular tissue samples (VI, Table 3).

Serine proteinase inhibitors

α_1–antitrypsin

α_1–antitrypsin–immunoreactive cells were mononuclear cells, but were only rarely found in the interface tissue samples, the pseudocapsular tissue samples, and the control synovial tissue samples (IV, Table 3).

The level of α_1–antitrypsin content was higher in the interface tissue samples than that of pseudocapsular tissue samples and control synovial tissue samples ($p=0.01$). The level of α_1–antitrypsin in the pseudosynovial fluid sample was the highest (IV, Table 3).

Immunoblot analysis showed marked bands of immunoreactive α_1–antitrypsin in the pseudosynovial fluid sample and moderate bands in the interface tissue sample, the pseudocapsular tissue sample and the control synovial tissue sample, corresponding to the α_1–antitrypsin control in the 50–60 kDa (IV).

α_1–antichymotrypsin

α_1–antichymotrypsin– positive cells were mononuclear cells, but were only rarely found in the periprosthetic interface tissue samples, the THR pseudocapsular tissue samples, fibrous area, and the control capsular tissues (III, Table 3).

α_1–antichymotrypsin content was detected in all samples. α_1–antichymotrypsin content was higher in the pseudosynovial fluid tissue samples than in any other samples ($p<0.01$; III, Table 3).

Immunoblot analysis also revealed bands of immunoreactive α_1–antichymotrypsin in the pseudosynovial fluid samples, weak to moderate bands in the interface, pseudocapsular tissue and control capsular tissue samples. Immunoreactive α_1–antichymotrypsin bands were observerd in 65–70 kDa area, which corresponded to the positive control, but several positive bands were also seen in 70–110 kDa area. In addition, another lower molecular weight band in the 50–60 kDa area was observed in solid tissue samples and fluid samples (III).

PAI–1 and PAI–2

All interface tissue samples and pseudocapsular tissue samples were stained for PAI–1. Compared to staining for uPA and tPA, larger areas were stained for PAI–1, especially in the interface tissue samples. PAI–2 staining was not as intense or extensive as for PAI–1, with only one pseudocapsule and three interface tissues showing slightly positive staining. Comparison of consecutive slides stained for uPA and PAI–1 revealed neighboring areas being positive for inhibitor and activator, but also with areas being positive for both(VI).

PAI–1 was found in much higher concentration than that of PAI– 2 both in the interface and pseudocapsular tissue samples. The level of PAI–1 was higher in the interface tissue samples ($p=0.0001$) and pseudocapsular tissue samples ($p=0.0001$) when compared to that of control capsular tissue samples. PAI–2 level was also higher in the interface tissue samples ($p=0.04$) and pseudocapsular tissue samples ($p=0.02$) when compared to that of control capsular tissue samples (VI, Table 3).

Type of prostheses and fixation

The effect of type of prostheses (Co-Cr-Mo versus Ti-Al-V) and fixation (cemented versus cementless) on immunohistochemical analysis of MMPs and elastase, on gelatinase activities (MMP–2 and MMP–9), and on elastase activity was also reviewed.

There was no statistical difference in the MMP–2 type and MMP–9 type gelatinolytic activities in different types of prosthetic fixation (cemented versus cementless) or types of alloy (Co-Cr-Mo versus Ti-Al-V; II).

Elastase activity as well as the number of elastase–immunoreactive cells was clearly higher both in the interface tissue samples and the pseudocapsular tissue samples obtained from loose THRs with different types of fixation (cemented versus cementless) and types of alloy (Co-Cr-Mo versus Ti-Al-V) than those in the control synovial tissue samples (IV).

Discussion

Cellular host reaction to total hip prostheses

Although it is reasonable to assume that loosening of THR prostheses is primarily caused by cyclic mechanical loading combined with cellular host reaction to implants, molecular mechanisms of the biological response to implants are still unknown as is the mechanical triggering of such mechanisms (Burke et al. 1991, Goodman et al. 1992a, 1993a, 1993b, Horowitz et al. 1993, Kim et al. 1993, Santavirta et al. 1992, 1993a, Stiehl et al. 1991). This study is focused on clarification of neutral proteinases and their inhibitors in periprosthetic tissues in loose THRs to determine their role in the biological periprosthetic process which is associated with loosening of the implant. It is important in this context that these enzymes play a major role in connective tissue remodeling, and are capable of degrading the collagenous proteins which constitute the major extracellular matrix macromolecules of the periprosthetic tissues. Also, the proteinase–inhibitor balance is important in tissue destruction (Brikedal–Hansen et al. 1993, Werb & 1989, Woessner 1991).

Samples

In this study, the samples from the interface tissues between bone and implants, from the pseudocapsular tissues surrounding the artificial joints, and the pseudosynovial joint fluid samples were able to be obtained in revision operations performed for loose THRs, whereas, because of ethical reason, it was not possible to obtain corresponding control samples from well fixed and noninfectious THRs.

Initially, capsular tissues (including the synovium) were obtained from fractured hip joints and these were used as controls in the immunohistochemical studies of MMPs. However, the capsular synovial tissues from fractured hip joints revealed elevated serine proteinase, cathepsin G level due to influx of inflammatory cells as the result of acute traumatic injury (Takagi et al. 1992). This result made it difficult to evaluate as to which extent the proteinase levels in periprosthetic tissues were elevated when compared to capsular synovial tissues of hip joints, although immunohistochemical analysis demonstrated increased immunoreactive cells of MMPs in the periprosthetic tissues when compared to capsular tissues from fracured hip joints. Thus, noninflammatory knee synovial tissues were considered as suitable control samples in such context (II–IV).

In this study model, a cause–and–effect relationship between the chemical and biological results obtained, and their role in implant loosening remains speculative. As yet, it has not been possible to study samples from well fixed and technically well functioning, noninfected THRs at comparable time intervals with loosened cases.

Matrix metalloproteinases and their inhibitors in loose THRs

The results presented immunohistochemically (I) demonstrate that certain MMPs (MMP–1, MMP–2, MMP–3, MMP–9) are induced in the interface tissues of loose THRs. Not only monocyte/macrophages but also fibroblasts and vascular endothelial cells synthesized MMPs both in the interface and pseudocapsular tissues. These findings indicate that periprosthetic tissue represents activated connective tissue involved in pathological matrix degradation resulting in prosthesis loosening, and tissue remodeling.

This immunohistochemical MMP profile suggested that MMP–1 type collagenase, not MMP–8, might play a critical role as an initiator in connective tissue remodeling and destruction, because it was capable, after in vitro activation with phenylmercuric chloride (Hasty et al. 1990), of degrading triple helical type I and III collagen monomers which constitute the major extracellular macromolecules of periprosthetic connective tissue beds. Subsequent structural degradation of extracellular macromolecule, denatured collagen, is mainly mediated by MMP–2 and MMP–9, and further also other MMPs such as MMP–3 (Birkedal–Hansen et al. 1993, Collier et al. 1988, Hibbs et al. 1985, Werb 1989, Wilhelm et al. 1989, Woessner 1991).

Collagenolytic and gelatinolytic potentials were confirmed biochemically by using HPLC and synthetic DNP–S degradation test (V). Collagenolytic activity refers to ability to degrade collagen, which can be mediated by fibroblast type collagenase (MMP–1), and gelatinolytic activity refers to ability to degrade gelatin (denatured collagen), which is probably mainly mediated by 72 kD gelatinase (MMP–2) and 92 kD gelatinase (MMP–9), both which were in accordance with immunohistochemical result (I).

Kim et al. (1993) reported that gelatinase levels

were elevated in the supernatant of organ culture derived from interface tissues of aseptic loosening, but the type of proteinases responsible for degradation of gelatin was not analyzed yet. The results presented by zymographic methods (II) demonstrate that i) MMP–2 type gelatinase/type IV collagenase production was highly elevated in the interface tissue and pseudocapsular tissue samples from loose THR endoprostheses compared to the control samples, ii) MMP–9 type gelatinase/type IV collagenase was induced in the interface tissue and pseudocapsular tissue samples but not in the control samples, iii) MMP–9 levels were higher in the interface tissue samples than in the pseudocapsular tissue samples.

Stromelysin–1 (MMP–3), stromelysin–2 (MMP–10) and pump–1 (MMP–7) as well as MMP–2 and MMP–9 have been reported to be able to degrade gelatin (Birkedal–Hansen et al. 1993, Werb 1989, Wilhelm et al. 1987, 1989). In this study, gelatinolytic activities corresponding to both MMP–2 and MMP–9 were identified. Whereas, gelatinolytic potential corresponding to molecular weights of MMP–3 (57 kDa), MMP–7 (28 kDa) and MMP–10 (57 kDa) was not detected by the zymographic method (II). However, other type of gelatinolytic MMPs, including membrane type MMPs, should be further evaluated by more sophisticated methods.

In this study, gelatin and type IV collagen were degraded even in the absence of organomercurials such as phenylmercuric chloride (II). Although *in vitro* activation by SDS could be excluded (Stricklin et al. 1983b, Werb 1989), it is more likely that endogenous activators (e.g., stromelysin and plasmin) contained in the periprosthetic tissue activate the latent collagenase proenzymes in situ (Ito et al. 1988, Konttinen et al. 1988, Ogata et al. 1992, Woessner 1991). Such an activation of enzymes in the periprosthetic tissues might lead to an imbalance between MMPs and tissue inhibitors of metalloproteinases *in loco* (Goldberg et al 1989, Goldberg et al. 1992, Ostheus et al. 1992, Woessner 1991).

Immunohistochemical analysis disclosed TIMP–1 in pseudosynovial lining cells, and often in monocyte/macrophages, endothelial cells and fibroblasts both in the interface and pseudocapsular tissues around loose endoprostheses. Pseudosynovial lining cells expressed moderate to marked immunoreactivity to TIMP–1, whereas staining of monocyte/macrophages, endothelial cells and fibroblasts was weak to moderate (V). The findings indicate that these cells can produce TIMP–1 but TIMP–1 expression may vary in different cells, perhaps depending on the degree of inflammation (Kodama et al. 1989).

The results (V) also demonstrate that low TIMP–1 level combined with high % degradation of synthetic DNP–S seems to lead to relatively high collageno– and gelatinolytic activity in the interface tissues around loose endoprostheses. Overwhelming production of MMPs in relation to TIMP–1 might occur *in situ*, which leads to enhancement of proteolytic processes and to weakening of periprosthetic connective tissue (Santavirta et al. 1993a, I, II).

Serine proteinases and their inhibitors in loose THRs

Elastase and cathepsin G, members of the serine proteinases, are considered as one of the modulators of inflammation and connective tissue remodeling and are able to act on extracellular matrix components at physiological pH prevailing in the extracellular space (Werb 1989). However, contribution of elastase–α_1–antitrypsin and cathepsin G–α_1–antichymotrypsin balances to activated proteolytic cascade in loose THRs *in situ* has not been previously studied.

In this study, immunohistochemical analysis (IV) demonstrated the tissue localization of elastase–immunoreactive cells and increased number of elastase–immunoreactive cells both in the interface tissues and the pseudocapsular tissues obtained from patients with loose hip prostheses when compared to that of control synovial tissues. This suggests that monocyte/macrophage– like mononuclear cells (and occasional PMNs) contain or produce elastase around loose hip prostheses.

Also, elastase activity was detected in periprosthetic tissue extracts in accordance with the results of immunohistochemical analysis (IV). The elastase activity was higher both in the interface tissues and the pseudocapsular tissues than that in the control synovial tissues. The presence of elastase in the periprosthetic tissues was also confirmed by the immunoblot analysis (IV).

The present results of cathepsin G (III) demonstrate the cellular localization and increased activity of cathepsin G in the tissues obtained from hips with loose endoprostheses. Although scatterd PMNs in or around blood vessels showed intense immunoreactivity to cathepsin G, immunohistochemistry combined with morphological evaluation and cell typing clearly demonstrated that monocyte/macrophages and fibroblasts were the main cathepsin G–positive cells in tissues around loosening endoprostheses. Immunoreactive monocyte/macrophages and fibroblasts were the major cells *in situ* whereas PMNs formed less than 1 % of all local cells. Although it has been earlier reported that cathepsin G is produced and / or stored

by PMNs and monocyte/macrophages (Campbell et al. 1989), the present data suggest that activated fibroblasts may also produce and / or contain cathepsin G.

Cathepsin G activity was detected in periprosthetic tissue extract samples too, which confirms the immunohistochemical results (III). The activity was higher in the interface tissue samples than in the pseudocapsular tissue samples. The pseudocapsular tissue samples consisted of highly cellular zones and and less cellular ones consisting mainly of fibroblasts. The proportion of the fibrous area in the pseudocapsular samples was larger than that found in the interface tissue samples, which might be the reason for the difference of the cathepsin G activity in these two tissue components. However, as the total volume of the pseudocapsular tissue sample is larger than that of the interface tissue sample *in situ*, the pseudocapsular tissue sample might be also important as a source of proteolytic enzymes. Cathepsin G activity was higher in both periprosthetic tissues than in the control capsular tissues and the pseudosynovial fluid samples. This suggests that periprosthetic tissues in loose THRs contain high amounts of cathepsin G, uninhibited by endogenous inhibitors, and thus, cathepsin G would probably be involved in loosening of the endoprostheses.

In contrast with the results of elastase and cathepsin G immunohistochemistry, α_1–antitrypsin and α_1–antichymotrypsin–immunoreactive cells were quite few in all the tissue samples, though immunoblot analysis and nephelometric immunoassay revealed the existence of α_1–antitrypsin and α_1–antichymotrypsin (III, IV). This could be explained by the small molecular size of α_1– antitrypsin (52 kD) and α_1–antichymotrypsin (68 kD) compared to that of the serine proteinase inhibitor, α_2–macroglobulin (725 kD).

The small molecular weights enable α_1–antitrypsin and α_1–antichymotrypsin to permeate into the interstitial space consisting of serum and synovial fluid and to inhibit the proteinase in inflammatory conditions (Travis et al. 1978, Werb 1989, III, IV), where it may participate in enzyme–inhibitor complex formation, which does not occur with the 725 kDa α_2– macroglobulin (Werb 1989).

Furthermore, immunoreactive bands, which did not correspond to those seen in the positive cathepsin G and α_1–antichymotrypsin controls, were also observed in immunoblot analyses of the tissue and pseudosynovial fluid samples. These bands might represent enzyme–inhibitor complexes or polymeric enzyme forms (Travis et al. 1978). Immunoreactive α_1–antichymotrypsin bands in 50–60 kDa area in the samples might represent the inactive form due to modification by proteolytic enzymes in the samples (Abbink et al. 1993, Sorsa et al. 1993, Travis et al. 1978).

One of the activation mechanism implicated for MMP–1 is the plasminogen activation system (Werb et al. 1977, Leloup et al. 1991). The inactive precursor of plasmin, plasminogen, occurs in the plasma and is activated by uPA and tPA. Focalized activation of plasmin by this mechanism is the starting point of a cascade activating collagenases and thus could lead to tissue destruction and loosening of the prostheses. The uPA, tPA, PAI–1 and PAI–2 constitute one of the main system regulating and focalizing extracellular matrix degradation *in vivo* through regulation of collagenase activation (He et al. 1989, Thomson et al. 1989, Vassalli et al.1991, Pelletier et al. 1992).

In this study, the presence of the plasminogen activating system, which is capable of activating collagenases and inducing tissue destruction including bone resorption (Thomson et al. 1989, Vassalli et al. 1991) was demonstrated by immunohistochemistry and ELISA (VI). The antigen levels in the periprosthetic tissues with high uPA and PAI–1 together with low tPA compared to the control capsular tissue samples, is similar to the differences reported for malignant and normal colon mucosa breast tissues (Jänicke et al.1991, Sier et al. 1991). The parallel findings to malignant tumor show that the tissue around loose THRs may have a high destructive capasity. It is interesting that although the PAI–1 antigen concentration was high compared to the activators, activation may still have take place on the cell surface in smaller compartments secluded from the inhibitor (Saksela and Rifkin 1988). Similar findings have been reported in invasive cancer, and is correlated to poor prognosis (Schmitt et al. 1991). This suggests that PAI–1 is an integral part of the aggressivity of the plasminogen activation system. It is possible that the aggressive periprosthetic tissues would, without PAI mediated protection, destroy themselves and thereby put an end to the destructive process. PAs may also be produced by osteoblasts and osteoclasts as a part of the periprosthetic bone resorption (Thomson et al. 1989, Grills et al. 1990, Leloup et al. 1991). PAI–1 could thus represent a form of self defense against proteolysis for the interface tissues as seen in invading carcinomas, which express PAs at the invasion zone and PAIs in the tumor tissue (Buø et al.1993).

This study also demonstrated that PAs were expressed almost exclussively by monocyte/macrophages with intracellular particles (VI). Monocyte/macrphages phagocytosing particulate debris also produce IL–1 (Glant et al. 1993), which could thus have been at least one of the cytokines mediating the increased uPA and PAI–1 production observed in

the present materials (Michel and Quertermous 1989, Pelletier et al. 1990).

Role of pseudocapsular tissues in loose THRs

Around loose artificial joints, regenerating capsular tissues also called the "pesudocapsular tissues", can be recognized in revision operations (Kim et al. 1993, Santavirta et al. 1993b). In pseudocapsular tissues, monocyte/macrophages, which were CD11b, CD68 and nonspecific esterase positive, fibroblasts, which were positive for proline 4–hydroxylase positive, and perivascular mononuclear cell infiltrations of mainly CD11/CD68 phenotype with CD4 positive cells, were observed, which suggested local host reaction to implants (Santavirta et al. 1993b). However, the precise role of pseudocapsular tissues has not been clarified yet.

It was of particular interest to observe that induction of immunoreactive MMP–positive cells (MMP–1, 2, 3 and 9) as well as the presence of elastase and cathepsin G–positive cells was not only confined to the interface tissues interposed between bone and implants but was also seen in the pseudocapsular tissue around prostheses (I). It appeared interesting that induction of DNP–S sensitive collageno– and gelatinolytic activity as well as elastase and cathepsin G activities was not only confined to the interface tissues interposed between bone and implants but was also seen in the pseudocapsular tissue (III, IV, V). In addition, in the zymographic analysis (II), induction of MMP–9 and elevated MMP–2 level were not confined to the interface tissues interposed between bone and implants but were also seen in the pseudocapsular tissue around the loose endoprostheses. The present study (I) demonstrated clearly the presence of MMPs in the periprosthetic local reactive inflammatory tissues and it appears evident that the MMPs participate in the biological implant loosening process (Santavirta et al. 1993b, I).

Kim et al. (1993) reported low gelatinase levels in the supernatants of organ cultures derived from pseudocapsular tissues. The discrepancy between their results and the ones presented here might result from the difference between the tissue extraction method, which directly reflects *in vivo* state, and the organ culture method, which is affected by *in vitro* circumstances. Once the tissues are transferred into *in vitro* systems, cellular responses including enzyme and inhibitor production could be modified.

The proteolytic enzyme response of the interface tissues could directly weaken the periprosthetic tissues. The wear debris found in the pseudocapsular tissues may induce cellular host response and proteolytic activation (Santavirta et al 1993b, Stricklin et al. 1983b). Thus, the pseudocapsular tissues could contribute to the loosening via production of MMPs into the synovial fluid.

Role of pseudosynovial fluid in loose THRs

As a local host reaction, pseudosynovial fluid in the artificial joint. Recently, it clearly demonstrated that THR causes an adaptive tissue response associated with formation of fibroblast–like B type lining cells which are able to synthesize and secrete hyaluronate (Saari et al. 1993, Santavirta et al. 1993b). However, the biological role of the pseudosynovial fluid is still relatively unknown.

Although it is difficult to compare exactly solid tissue samples with pseudosynovial fluid samples due to the difference of material processing, it is of particular interst that the relatively high content of TIMP–1 in the pseudosynovial fluid may explain the low DNP–S sensitive collageno– and gelatinolytic activity. α_2–macrogloblin (Mr 725K) in the fluid samples may further suppress the collageno– and gelatinolytic activity, but would be unable to permeate into the interstitial space (He et al. 1989), where the more small molecular inhibitor TIMP–1 (Mr 28 K) as well as other TIMPs are the main inhibitors (Clark et al. 1993).

Also, it is noteworthy that the THR pseudosynovial fluid samples contained only low elastase and cathepsin G activities. However, laser nephelometric and immunoblot analyses revealed not only the existence of these enzymes but also high contents of α_1–antitrypsin and α_1–antichymotrypsin in the pseudosynovial fluid samples. It is possible that the protease inhibitors, α_1–antitrypsin and α_1–antichymotrypsin, as well as α_2–macrogloblin (Werb 1989), may inhibit elastase and cathepsin G activities, respectively, in the pseudosynovial fluid, which leads to a low activity in spite of the presence of such enzymes (Abbink et al. 1993, Beatty et al. 1980). This has been reported to be the case in osteoarthritis and rheumatoid arthritis, which further supports the hypothesis that the presence of inhibitors may explain the difference of elastase and cathepsin G activities between tissues and pseudosynovial fluid (Virca et al. 1984).

Thus, pseudosynovial fluid could be produced to prevent the unfavourable elevation of proteolytic enzymes *in loco* as a local host response to implant reactions.

Type of prosthesis and fixation

The effect of the type of prostheses (Co-Cr-Mo versus Ti-Al-V) and fixation (cemented versus cementless) on immunohistochemically analysed of MMPs and elastase, and on gelatinase (MMP–2 and MMP–9) and elastase activities was of interest.

Although the number of the examined cases was too small for relevant statistical analysis, there was no indication that the chemical properties of the implants would have had a specific effect on the enzyme biology studied here. Once a cellular host reaction occurs in the periprosthetic tissues and causes loosening, it appears to induce neutral proteinases regardless of type of fixation or prosthetic materials and the possible differences are not measurable with the present techniques.

Periprosthetic foreign body reaction and osteolysis

Periprosthetic foreign body reaction has been considered as common phenomenon observed in aseptic loosening (Harris et al. 1976, Santavirta et al. 1990). In this study, as the local host reaction, imbalance of neutral proteinases and their inhibitors around loose prostheses were demonstrated in periprosthetic inflammatory connective tissues. It seems that such reaction may impair periprosthetic extracellular matrix remodelling, which can also provide much more suitable location of particle–related foreign body reaction. Enhanced foreign body reaction can produce inflammatory mediators, such as IL–1, IL–6, TNF–α, PDGF, and PGE_2 (Goldring et al. 1983, Howie et al. 1993, Jiranek et al. 1993, Horowiz et al. 1993, Hynes et al. 1993, Santavirta et al. 1994), which lead to activation of osteoclasts and bone resorption around prostheses as well as activation of the proteinases in periprosthetic connective tissues. Activated osteoclast can also produce enzymes, including MMP–9 (Tezuka et al. 1994), which can degrade the denatured bony extracellular matrices, which may be also attacked by excessive neutral proteinases, matrix metalloproteinases and serine proteinases, existed in the adjacent periprosthetic inflammatory area, thus may lead to advanced osteolysis or implant loosening.

Regulation of unfavorable local host reaction to hip prostheses

Neutral proteinases locally produced and uninhibited by endogenous inhibitors may, together with a cyclic mechanical loading, take share in the loosening of hip prostheses. The clinical relevance of this study and of deeper understanding of the mechanisms of proteolysis involved in the loosening may help in the development of synthetic and recombinant modulators of proteinases that may prevent the degradation of the periprosthetic connective tissue bed. Deeper understanding of the proteolytic cascade in the loosening may in future lead to modulation of the biological process connected with prosthetic loosening. Proteinases can be chemically inhibited and it would appear possible to modulate activated enzymes by synthetic and recombinant reagents in pathological conditions (Breedveld et al. 1990, Gloub et al. 1992, Ingman et al. 1993, Knight et al. 1992, Lauhio et al. 1993, 1994, Nagai et al. 1992, Scarpellini et al. 1994, Sorsa et al. 1993, Suomalainen et al. 1992, Wewers et al. 1987, Vincentti et al. 1994, Yu et al. 1992).

General conclusions

I. It was demonstrated that MMPs (MMP–1, MMP–2, MMP–3, MMP–9) were induced in cells in interface tissues between bone and implants, which contained a large number of MMP–immunoreactive cells compared to that which was found in the synovial capsular tissues obtained from fractured hips. It is apparent that the conditions in THRs induce a wide spectrum of MMPs capable of degrading extracellular matrix macromolecules and mediating prosthetic loosening. Collageno– and gelatinolytic activity, MMP–2 and MMP–9 activity, which were not inhibited, were elevated in the interface tissues, which was confirmed by the immunohistochemical analysis.

II. This study demonstrated the presence, tissue localization and content of elastase and cathepsin G in periprosthetic interface tissues *in situ* around loose THR endoprostheses, suggesting that uninhibited elastase and cathepsin G in the tissues may also play a significant role in weakening of the periprosthetic connective tissue and lysis of the bone around implants and thus cause loosening of the endoprostheses.

III. Cellular host response involving elevated collageno– and gelatinolytic activity, MMP–2 and MMP–9 activities, elastase and cathepsin G activities, which were not supressed by endogenous inhibitors, was also observed in pseudocapsular tissues around loose prostheses as well as interface tissues between bone and implants, which suggested the possible role of pseudocapsular tissues as not only regenerating capsules, but also a source of proteolytic enzymes which induce periprosthetic weakening.

IV. The presence of the plasminogen activation cascade (uPA, tPA, PAI–1 and PAI–2) was demonstrated in the periprosthetic tissues. This system generates focalized activation of plasmin that may be the starting point of a cascade activating collagenases and thereby tissue destruction and loosening of the prostheses.

V. Pseudosynovial fluid obtained in revision operations contained relatively high levels of inhibitors (TIMP–1, α_1–antitrypsin and α_1–antichymotrypsin) associated with low collageno– and gelatinolytic activity, elastase activity and cathepsin G activity. The role of the pseudosynovial fluid could be prevention of the unfavorable elevation of proteolytic enzymes *in loco* as a local host response to the implant.

VI. Effect of type of prostheses (Co-Cr-Mo versus Ti-Al-V) and fixation (cemented versus cementless) on immunohistochemical analysis of MMPs and elastase, on gelatinase activities (MMP–2 and MMP–9), and on elastase activity was studied, but the type of prostheses and fixation did not affect the enzymes. Once a cellular host reaction occurs in periprosthetic tissues and causes loosening, it appears to induce neutral proteinases regardless of type of fixation or prosthetic materials.

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