Gelatinolytic and Collagenolytic Activity in Periprosthetic Tissues from Loose Hip Endoprostheses

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ABSTRACT. ***Objective.*** To study the contribution of different members of the metalloproteinases (MMP) family in gelatinolytic and collagenolytic potential, namely dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg (DNP-S) sensitive proteolytic activity, in loose total hip arthroplasty (THA) endoprostheses.

Methods. Periprosthetic tissues and fluid samples were collected from patients subjected to hip endoprosthesis replacement. DNP-S sensitive proteolytic activity was evaluated by the degradation of synthetic DNP-S and reverse phase high performance liquid chromatography, while gelatinolytic activity was assessed by gelatin zymography. The isolation and separation of gelatinases was performed by gelatin- and concanavalin A-Sepharose affinity chromatographies and the identification of collagenases by immunoblot analysis.

Results. High gelatinolytic activity was observed in all periprosthetic tissue extracts and fluid samples. All samples also exhibited DNP-S degrading activity, without pretreatment by activating agents. Upon fractionation of MMP by gelatin-Sepharose affinity chromatography it was found that the gelatin-unbound collagenases are exclusively responsible for DNP-S degrading activity. Activated species of both MMP-1 and 13 were detected in most samples, but not the soluble form of MT1-MMP. Separation of gelatinases from each other and treatment with 4-aminophenylmercuric acetate (APMA) revealed that both enzymes mainly existed in complex with tissue inhibitor of metalloproteinase (TIMP).

Conclusion. MMP-1 and MMP-13, which exist in activated form, could be responsible for the DNP-S-degrading activity in periprosthetic tissues and fluids, while the gelatinases do not contribute in this potential, since they mainly exist in complex with TIMP. The 2 collagenases may play a key role in the loosening of THA endoprostheses. (J Rheumatol 2001;28:1319–29)

Key Indexing Terms:

METALLOPROTEINASES	GELATINASES	COLLAGENASE-1
COLLAGENASE-3	TOTAL HIP ARTHROPLASTY	LOOSENING

The biocompatibility of total hip arthroplasty (THA) prostheses has been a subject of debate for 3 decades. It appears that loosening of well inserted prostheses is usually produced by a cascade of mechanical and biological events. These sequences of events consist of fragmentation and wear of the implanted materials and subsequently the release of implant particles into the periprosthetic tissues, evoking a foreign-body granulomatous reaction and activation of cells to produce a variety of cytokines, growth factors, and proteolytic enzymes. Through complex mechanisms, these cell products cause periprosthetic osteolysis that exceeds the reparative capacity of the fibrous and osseous tissues, resulting in loosening of the components from the skeleton[1-14]. The role of proteolytic enzymes in this process appears to be very important. Among them, the extracellular matrix metalloproteinases (MMP) play a significant role[15-24].

MMP make up a large family of over 20 zinc dependent metallopeptidases, which are able to degrade almost all the extracellular matrix components and are thought to play a key role in the remodelling activity of cells, associated with both physiological and pathological manifestations[25-28].

MMP-1, MMP-8, and MMP-13, members of the MMP group of interstitial collagenases, cleave the collagen triple helix of type I and III at a single locus to yield characteristic 3/4 and 1/4 cleavage products[29-31]. MMP-13, the more recent member of the group, also degrades type II collagen and gelatin[31]. Degraded collagens are denatured into gelatins at body temperature and gelatins as well as type IV collagens are digested by gelatinases /type IV collagenases (MMP-2 or gelatinase A of 72 kDa and MMP-9 or gelatinase B of 92 kDa), another group of the MMP family[32-35]. The 2 gelatinases are unique among MMP in that they are

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bound with tissue inhibitor of metalloproteinase (TIMP) in both the latent and activated form, the MMP-9 proenzyme with TIMP-1 versus the MMP-2 precursor with TIMP-2[35,36]. The presence of TIMP prevents the activation of the respective gelatinase proenzyme by 4-aminophenylmercuric acetate (APMA) or proteinases[37-41].

The presence of MMP-1, 2, 3, 9, 13, and MT1-MMP as well as TIMP-1 and TIMP-2 in periprosthetic tissues and fluid from loose THA endoprostheses has been established from many investigators[15,17,19-22,42-44]. In addition, the presence of other proteolytic enzymes, such as elastase, cathepsin G, and plasminogen activators, has been reported[45-47].

Several assays have been applied to evaluate the activity of individual MMP in periprosthetic tissue extracts or in conditioned media of periprosthetic tissue explant cultures, such as gelatin zymography and the degradation of native substrates followed by identification of degradation products[15,18,19,23,24]. Since these assays were performed in crude extracts or conditioned media, the observed degradation of the substrates may be due to the synergistic effect of different proteolytic enzymes present in extracts or conditioned media. In addition, gelatinolytic activity determined by gelatin zymography may be due to proenzymes of gelatinases as well as to their activated forms, which, however, could exist in complex with TIMP and upon electrophoresis in the presence of sodium dodecyl sulfate (SDS) are dissociated[38-40]. The synthetic substrate 2,4-dinitrophenyl-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg (DNP-S) was used by Tagaki, *et al*[16] to determine gelatinolytic and collagenolytic activity in crude extracts of periprosthetic tissues uninhibited by TIMP. However, it is known that DNP-S is cleaved in the same mode by interstitial collagenases and gelatinases[48–50].

We separated MMP, which are present in periprosthetic tissues and fluid, and investigated their contribution in DNP-S sensitive collagenolytic and gelatinolytic activity, uninhibited by endogenous inhibitors. This was done to evaluate the role of each MMP found in periprosthetic tissues and fluid in the cascade of matrix degradation that occurs in the loosening of THA endoprostheses.

MATERIALS AND METHODS

Materials. Concanavalin A-Sepharose 4B, gelatin-Sepharose 4B, protein A-Sepharose 4B, casein, DNP-S, APMA, and peroxidase conjugated anti-mouse IgG were purchased from Sigma Chemical Co. (St. Louis, MO, USA); gelatin was from BDH Chemicals Ltd. (Poole, England). All other chemicals used were analytical grade. Monoclonal antibodies against human MMP-1 (clone 41-1E5) and MT1-MMP (clone 114-6G6) and human TIMP-1 and TIMP-2 were purchased from Chemicon, Temecula, CA, USA. Mab against human MMP-13 (clone 181-15A12) were kindly provided by Dr. C. López-Otin (Universidad de Oviedo, Departamento de Biologia Funcional, Oviedo, Spain). Polyclonal antibodies against MMP-2 and MMP-9 were the generous gift of Dr. P. Koolwijk (Gaubius Lab. TNO - PG, Leiden, The Netherlands).

Patients and samples. Five samples of interface tissue around loose femoral components were obtained in revisions performed for aseptic total hip replacement loosening. Samples of pseudocapsular tissue and pseudosynovial fluid from the loose prostheses were obtained in 4 and 2 cases, respectively. Specimens were from 3 women and 2 men with a mean age of 68.6 years (range 64–72). All THA were originally performed for primary osteoarthritis. The fixation was cemented in 3 patients and cementless in 2. Radiographs showed focal femoral osteolysis in 3 out of 5 patients. Immediately after aspiration from the joint pseudosynovial fluid samples were centrifuged at 12,000 g for 10 min at 4°C to remove cells and debris. All samples were kept at –80°C until use.

Tissue extraction and dialysis. Extraction of tissues and dialysis were performed as described[15,16] with minor modifications. In brief, tissue samples were minced into small pieces and homogenized with a Polytron homogenizer in extraction buffer (50 mM Tris-HCl, pH 7.5/10 mM $CaCl_2$/2 M KCl; 3 ml buffer/g tissue) containing the proteinases inhibitors 1 mM phenylmethanesulfonyl fluoride, 10 mM N-ethylmaleimide (NEM), and 10 mM benzamidine-HCl in an ice bath. After stirring for 1 h at 4°C, the homogenates were centrifuged at 10,000 g for 30 min at 4°C. The supernatants were adjusted to contain at final concentration 250 mM sucrose and then they were centrifuged at 100,000 g for 1 h at 4°C. The 100,000 g supernatants were exhaustively dialyzed against enzyme buffer (50 mM Tris-HCl, pH 7.5/10 mM $CaCl_2$/0.2 M NaCl/0.02% NaN_3), concentrated by ultrafiltration using PM-10 membrane (Amicon Corp., Beverly, MA, USA) to a final volume corresponding to 1 ml/g of initial tissue, and applied for the assays. Synovial fluid samples were diluted with extraction buffer (1:1), dialyzed against enzyme buffer for 24 h at 4°C, and also applied for the assays.

Enzyme separation. Gelatinases were separated from other metalloproteinases present in the extracts by gelatin-Sepharose affinity chromatography as described[38,39]. In brief, the samples in enzyme buffer were adjusted to contain 0.5 M NaCl and 0.1% Triton X-100 and were applied to a column of gelatin-Sepharose, equilibrated with 50 mM Tris-HCl, pH 7.5/0.5 M NaCl/0.02% NaN_3/0.1% Triton X-100 buffer. The gelatin-unbound MMP were eluted with equilibration buffer and the gelatin-bound gelatinases with the same buffer containing 1 M NaCl and 5% (v/v) dimethyl sulfoxide.

The 2 gelatinases were separated from each other by concanavalin A-Sepharose (Con A-Sepharose) affinity chromatography, according to Morodomi, *et al*[38]. The gelatin-bound pool from the above affinity chromatography, containing the MMP-2 and MMP-9, was dialyzed against 50 mM Tris-HCl, pH 7.5/0.15 M NaCl/10 mM $CaCl_2$/0.02% NaN_3 buffer, adjusted to contain 0.05% Triton X-100, and applied to a Con A-Sepharose column equilibrated with the same buffer containing 0.05% Triton X-100. The unglycosylated MMP-2 is not bound to Con A and was eluted in the flow-through fractions, while the bound MMP-9 was eluted with equilibration buffer containing at final concentration 0.6 M NaCl and 1 M methyl-α-D-mannopyranoside.

To remove any contamination of MMP-9 from the pool containing the MMP-2 and vice-versa, each of the 2 pools from Con A-Sepharose chromatography was subjected to further purification by immunoprecipitation, using polyclonal antibodies against MMP-9 and MMP-2, respectively, and protein A-Sepharose. Each pool was dialyzed against enzyme buffer, and the respective antibodies were added and incubated at 4°C for 48 h. Protein A-Sepharose gel was then added and the incubation continued for 20 h at 4°C. Postincubation the gel was removed by centrifugation and the supernatants were collected.

Gelatin zymography. The samples were preincubated at 37°C for 15 min in Laemmli sample buffer[51] and then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE)[51] under nonreducing conditions in 10% polyacrylamide gels, incorporating 1 mg/ml gelatin. After electrophoresis the gels were washed twice at room temperature for 15 min each time with 50 mM Tris-HCl, pH 7.5/5 mM $CaCl_2$/1 μM $ZnCl_2$/0.02% NaN_3 buffer containing 2.5% Triton X-100, followed by a 15 min wash with the same buffer containing 0.1% Triton X-100, and then incubated at 37°C for 20 h in the same buffer. Postincubation the gels were stained with 0.25% Coomassie

brilliant blue R-250 in 9% (v/v) acetic acid/45% (v/v) methanol, destained with 7% acetic acid/40% methanol, and kept in 7% acetic acid. Enzymatic activity was verified by computer analysis of the intensity of each gelatin lysis band. Gels were scanned on a digital scanner (Hewlett Packard, ScanJet 6100 C/T) using the program Corel Photo Paint 7 and image processing took place with the Image PC program.

Determination of collagenolytic and gelatinolytic activity using the synthetic substrate DNP-S and high performance liquid chromatography (HPLC). DNP-S degrading activity was measured as described[49]. The samples in enzyme buffer (total volume 10 μl) were incubated in the presence of 1.25 mM DNP-S at 37°C for 4 h. Adding equal volume of trifluoroacetic acid (TFA) solution 0.2% stopped the reaction and samples were centrifuged. Then 10 μl of supernatant was subjected to reverse phase HPLC using an HPLC system (Waters system controller 600E, Millipore Corp., Waters Chromatography, Bedford, MA, USA) equipped with a 4 × 250 mm Lichrospher 100RP-18 column (5 μm particle size, Merck, Darmstadt, Germany) to separate nondegraded DNP-S and its 2 degradation products, 2,4-dinitrophenyl-Pro-Gln-Gly (DNP-P) and Ile-Ala-Gly-Gln-D-Arg. The column was eluted with a gradient of 25–32% acetonitrile in 0.1% TFA, at room temperature, for 25 min, at a flow rate 1 ml/min. Column effluent was continuously monitored during the separation with a variable wavelength monitor (Waters photodiode array detector 991, Millipore Corp.) at 260 nm and recorded with an online printer (Waters Recorder 5200, Millipore Corp.) to detect nondegraded DNP-S and its product DNP-P. The retention time of DNP-S and DNP-P was determined in preliminary experiments by chromatography of DNP-S before and after digestion with human MMP-9 activated with trypsin[38]. The rate of degradation of DNP-S substrate was expressed as (DNP-P/DNP-S) × 100%. Given the initial amount of DNP-S, the amount of the DNP-P produced could be calculated. Thus, the specific DNP-S degrading activity was expressed as nmole DNP-P/μg protein.

Immunoblotting. Samples of gelatin-Sepharose unbound pool of tissue extracts and fluids with the same amount of protein were enriched in MMP by precipitation with $(NH_4)_2SO_4$ (60% saturation). The resultant precipitates were dissolved in Laemmli sample buffer[51], treated with 2-mercaptoethanol, and then subjected to SDS-PAGE[51] on 12% polyacrylamide gels. After electrophoresis the separated proteins were electrotransferred onto nitrocellulose membranes according to the method of Towbin, *et al*[52]. The free binding sites on the nitrocellulose membranes were blocked with 5% skim milk in 20 mM Tris-HCl, pH 7.4/150 mM NaCl buffer (TBS), containing 0.05% Tween-20 (TBS-T), at room temperature for 2 h. After three 15 min washes with TBS-T, the membranes were incubated with mouse monoclonal anti-human MMP-1 (3.5 μg IgG/ml) or MMP-13 (2 μg IgG/ml) or MT1-MMP (10 μg IgG/ml) in TBS-T, containing 1% skim milk, at 4°C for 16 h. After washing 3 times with TBS-T, they were incubated with peroxidase conjugated goat anti-mouse IgG at dilution 1:2000 in TBS-T containing 1% skim milk at room temperature for 2 h. Then the membranes were washed with TBS-T 3 times, once with TBS, and the immunoreacted proteins were detected by the enhanced chemiluminescence method, according to the manufacturer's instructions (Pierce, Rockford, IL, USA).

Treatment of samples with APMA. The samples were incubated in the presence of 1 mM APMA, dissolved in DMSO, in enzyme buffer (50 mM Tris-HCl, pH 7.5/0.2 M NaCl/10 mM $CaCl_2$ /0.02% NaN_3) at 37°C for 48 h and then subjected to gelatin zymography.

Protein determination. Protein content of samples was determined by the method of Bradford[53] using bovine serum albumin as standard.

Statistical analysis. Statistical analysis of the results was by Student nonpaired t test.

RESULTS

Gelatin-degrading activities in the periprosthetic tissue extracts and fluids. Samples of interface and pseudocapsular tissue extracts and pseudosynovial fluids, containing the same amount of protein, proMMP-9 from human polymorphonuclear leukocytes, and proMMP-2 from human synovial fibroblasts were subjected to gelatin zymography (Figure 1). Four major gelatin lysis bands of molecular mass 92, 83, 67, and 65 kDa appeared in all samples. In pseudosynovial fluid samples, however, the 65 kDa band appeared at considerably lower intensity (Figure 1C). In some samples 2 minor bands in the region of 45 kDa were also observed. The lysis bands of 67 and 92 kDa migrated in the gel in the same way as the control proMMP-2 and proMMP-9, respectively. Since all the samples contained equal amounts of protein, by measurement of total intensity from all lysis bands in each sample it was found that the specific gelatinolytic activity in the samples of each group of tissues and fluid was invariable between different patients. However, the specific gelatinolytic activity in pseudocapsular tissue samples was lower than in the interface tissue sam-

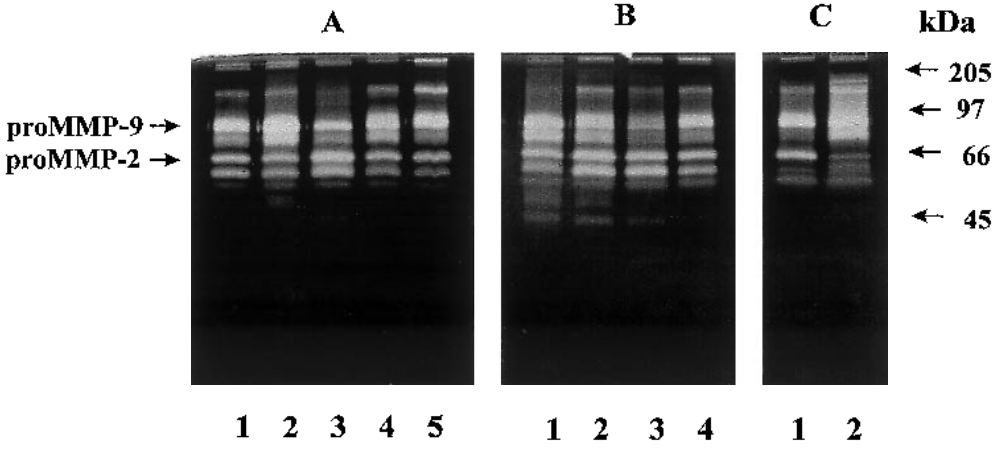

Figure 1. Representative gelatin zymography of interface (A), pseudocapsular (B) tissue extracts, and pseudosynovial fluids (C). All samples contained the same amount of protein and were run in duplicate.

ples (20%; $p < 0.05$). Even lower specific gelatinolytic activity was found in pseudosynovial fluid samples. Gelatinolytic activity in all samples was completely inhibited by developing the zymograms in the presence of 20 mM Na_2-EDTA (not shown), indicating that all lysis bands corresponded to metalloproteinases. The 67 and 92 kDa lysis bands represent MMP-2 and MMP-9 proenzymes, respectively. The 83 and 65 kDa lysis bands may represent activated forms of MMP-9 and MMP-2. That the 67 kDa lysis band may also represent, at least in part, the activated form of MMP-9 cannot be excluded. The 45 kDa lysis bands may represent MMP-3, as confirmed using casein zymography (not shown).

DNP-S-degrading activity in the periprosthetic tissue extracts and fluids. All samples tested exhibited DNP-S-degrading activity (Figure 2) without pretreatment by activating agents. The specific DNP-S-degrading activity was significantly higher in interface and pseudocapsular tissue samples compared to pseudosynovial fluid samples (Figure 3, column I). DNP-S-degrading activity was completely inhibited by 20 mM Na_2-EDTA or TIMP (not shown), indicating that it was due to MMP. The fact that this activity was expressed with no pretreatment of extracts and fluids with activating agents, such as organomercurials, indicates that the MMP preexisted in their activated forms and their levels exceeded those of TIMP.

To discriminate which MMP participates in the cleavage of DNP-S, the MMP in periprosthetic tissue extracts and fluids were separated by gelatin-Sepharose affinity chromatography. The 2 pools obtained (gelatin-bound and -unbound) were subjected to gelatin zymography (Figure 4) and tested for DNP-S-degrading activity. As shown in Figure 4 the vast majority of the gelatinolytic activity of tissue extracts and fluids were obtained in the gelatin-bound pool, indicating that all gelatin lysis bands, as observed (Figure 1), represent forms of gelatinases. Although this pool contained almost all the gelatinolytic activity, it exhibited no DNP-S-degrading activity (not shown). On the contrary, all DNP-S-degrading activity was obtained in the gelatin-unbound pool of all samples tested, since their specific DNP-S-degrading activity (Figure 3, column II) was almost the same, compared to that of the nonfractionated initial tissue extracts and fluids (Figure 3, column I). These results indicate that MMP in the extracts, other than gelatinases, are responsible for the degradation of DNP-S.

Identification of collagenases in tissue extracts and fluids. The presence of MMP-1, MMP-13, and in very low levels, MMP-8 in interface and pseudocapsular tissues and fluid, as well as the presence of a soluble type of MT1-MMP in pseudosynovial fluid, has been established[17,19-22,42-44]. Since the 3 collagenases and probably the MT1-MMP have the same specificity [cleave the peptide bond Gly-Ile(Leu) at a single specific initial cleavage site][29-31], it was assumed that these enzymes may contribute to DNP-S-degrading activity if

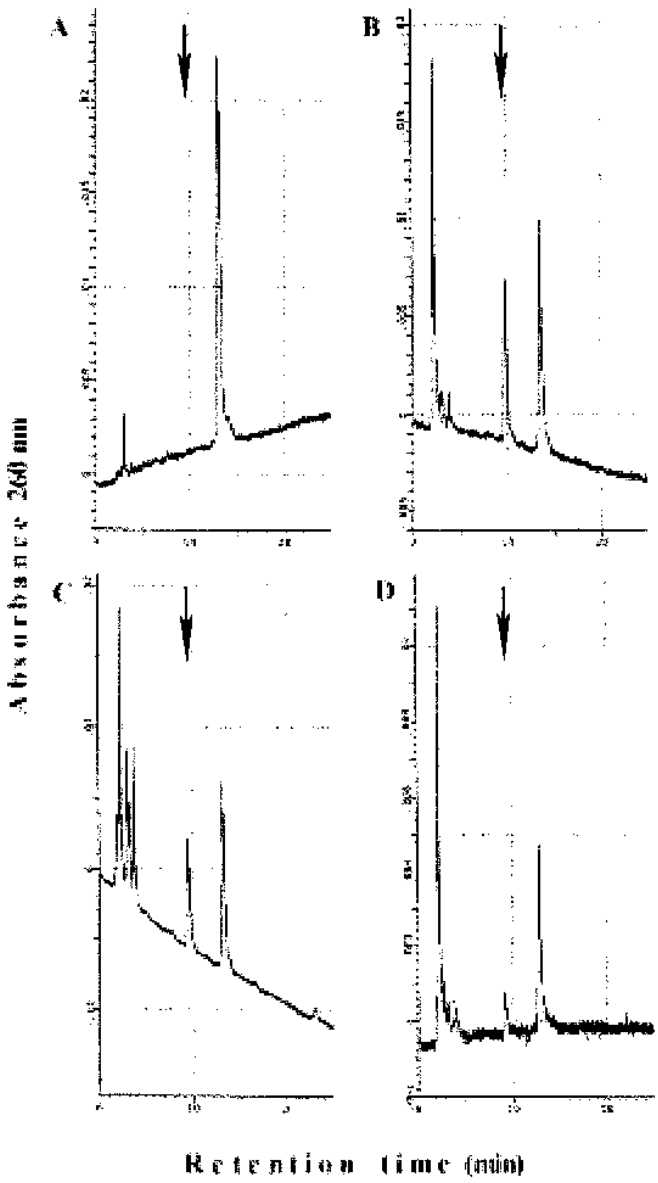

Figure 2. Monitoring of degradation of DNP-S by RP-HPLC in an area mode. (A) control without sample, (B) interface tissue, (C) pseudocapsular tissue, (D) pseudosynovial fluid. Arrow indicates retention time of DNP-P.

they are present in the samples tested. Thus, their presence in the gelatin-unbound pool of all samples was verified by immunoblot analysis using specific Mab that recognize only the proenzyme and the activated forms of the respective MMP (Figure 5).

A major immunoreactive band of molecular mass 52.5 kDa in all samples tested, and 2 more minor bands of molecular mass 43.5 and 45 kDa in most interface and pseudocapsular tissue samples, appeared when monoclonal anti-MMP-1 was used (Figure 5A). The major band probably represents the unglycosylated form of proMMP-1 and the 2 minor bands the proteolytically activated MMP-1 enzyme species of both glycosylated and unglycosylated forms.

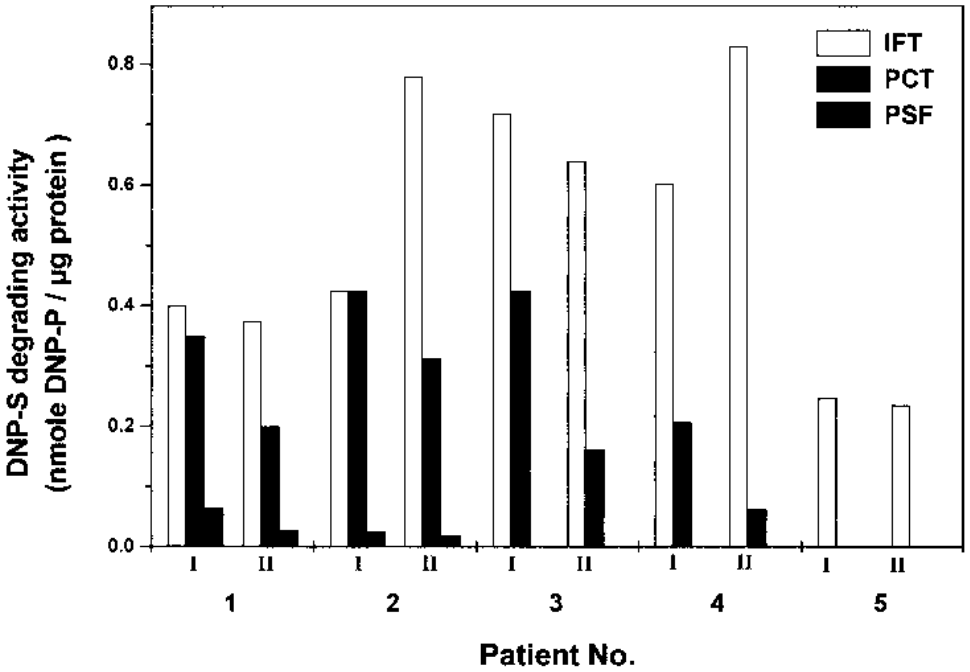

Figure 3. Specific DNP-S-degrading activity of interface and pseudocapsular tissue, and pseudosynovial fluid samples, measured by RP-HPLC. (I) initial extracts, (II) gelatin-Sepharose affinity chromatography unbound pools. IFT: interface tissue, PCT: pseudocapsular tissue, PSF: pseudosynovial fluid. Values are the mean of 2 independent experiments.

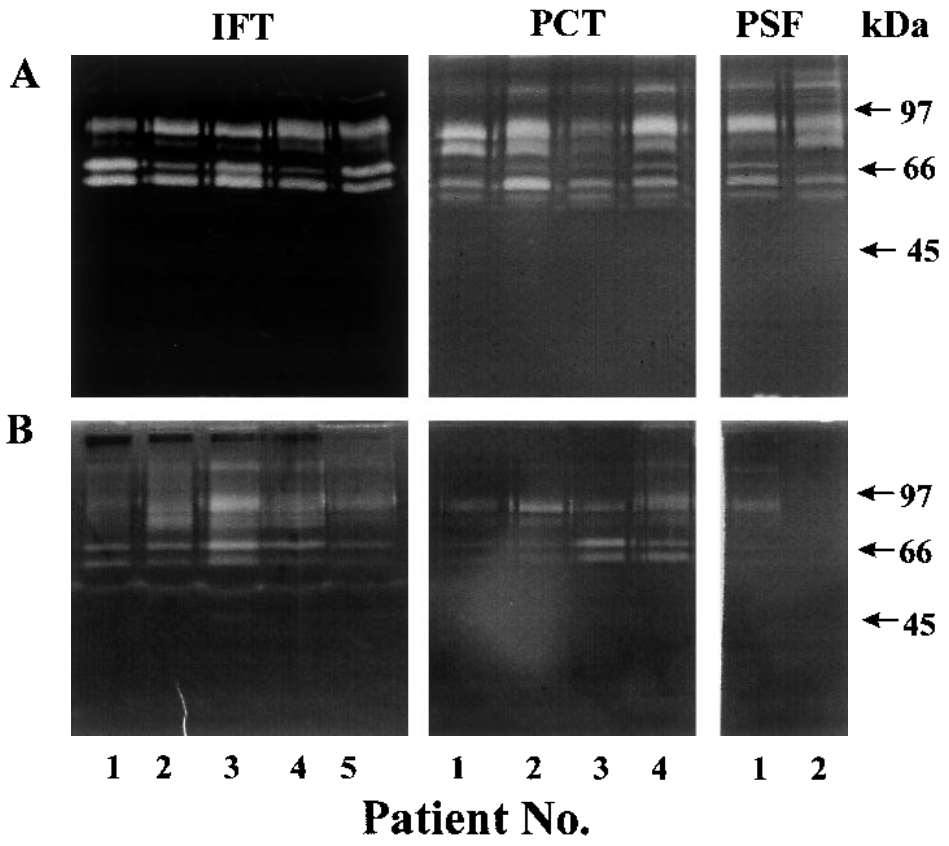

Figure 4. Gelatin zymography of the bound (A) and unbound (B) pool from gelatin-Sepharose affinity chromatography of interface and pseudocapsular tissue and pseudosynovial fluid samples. IFT: interface tissue, PCT: pseudocapsular tissue, PSF: pseudosynovial fluid.

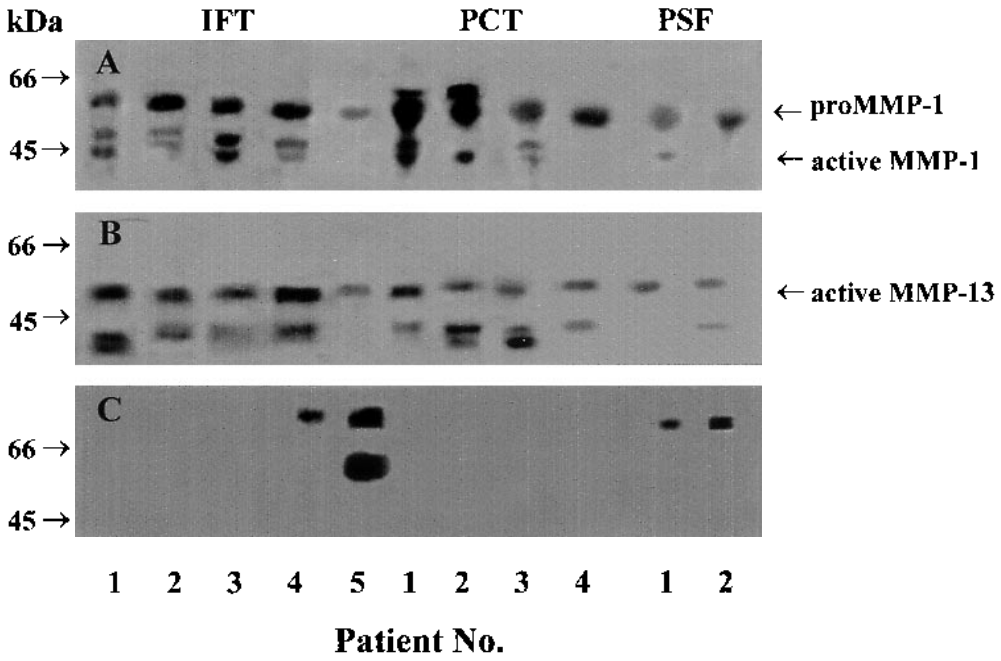

Figure 5. Western blotting of unbound pool from gelatin-Sepharose affinity chromatography of interface and pseudocapsular tissue and pseudosynovial fluid samples using Mab against MMP-1 (A), MMP-13 (B), and MT1-MMP (C). IFT: interface tissue, PCT: pseudocapsular tissue, PSF: pseudosynovial fluid.

Although activated species of glycosylated form were found in most samples, in only 2 samples was the respective proenzyme of 57 kDa detected (Figure 5A, PCT rows 1 and 2).

When monoclonal anti-MMP-13 was used, an immunoreactive band of molecular mass 48 kDa was found in all samples tested (Figure 5B), and in some samples 2 more bands of 38 and 34.5 kDa were also observed. The 48 kDa band may represent the fully activated form of MMP-13, while the other 2 lower molecular mass bands probably represent degradation products of this enzyme.

No immunoreactivity was observed in most samples tested when the monoclonal anti-MT1-MMP was used (Figure 5C). A 70/69 kDa duplex band appeared in only the 2 pseudosynovial fluid and in 2 interface tissue samples, and in addition a 56.5 kDa band appeared in one interface tissue sample, which probably represent the latent form and an intermediate proteolytically processed form of MT1-MMP, respectively.

Since MMP-8 has been detected in very low amounts in periprosthetic tissues and fluid, these results indicate that the responsible collagenases for DNP-S-degrading activity in periprosthetic tissue extracts and fluids are probably MMP-13 and MMP-1.

Existence of gelatinases in periprosthetic tissue extracts and fluids in complex or not with TIMP. The inability of gelatinases to degrade DNP-S, while their activated forms were observed in gelatin zymography (Figures 1 and 4), raised the question whether they existed in complex with TIMP. For this reason, the 2 gelatinases were separated from each other, as described in Materials and Methods, and subjected to gelatin zymography with and without treatment with APMA (Figures 6 and 7).

It was observed that the Con A-unbound pool of all the samples (Figure 6A) contained the 2 lysis bands of molecular mass 67 and 65 kDa. After treatment with APMA (Figure 6B) only the 65 kDa lysis band appeared, with intensity equal to the sum of the intensities of the individual 67 and 65 kDa bands before the treatment with APMA. This observation indicates that the 67 kDa lysis band was converted to 65 kDa, while the preexisting 65 kDa band remained unaltered. The 67 and 65 kDa bands represent the proMMP-2 and a converted form of MMP-2, respectively. The conversion of the 67 kDa band to 65 kDa by APMA and the observed stability of the preexisting as well as the resulting 65 kDa band, even after prolonged treatment with APMA, as reported[39], indicate that the MMP-2 in all samples existed in complex with TIMP-2. If proMMP-2 was TIMP-free, upon treatment with APMA it would be converted first to a 65 kDa form, which is unstable, and afterwards it would be self-degraded[39]. The same would be observed if the preexisting 65 kDa form corresponded to the TIMP-free activated form of MMP-2.

The Con A-bound pool of all the samples (Figure 7A) contained gelatin lysis bands of molecular mass 92, 83, and 67 kDa. After treatment with APMA (Figure 7B) the 92 kDa band disappeared, the intensity of the 83 kDa band increased, the sharp band of 67 kDa disappeared, and a new

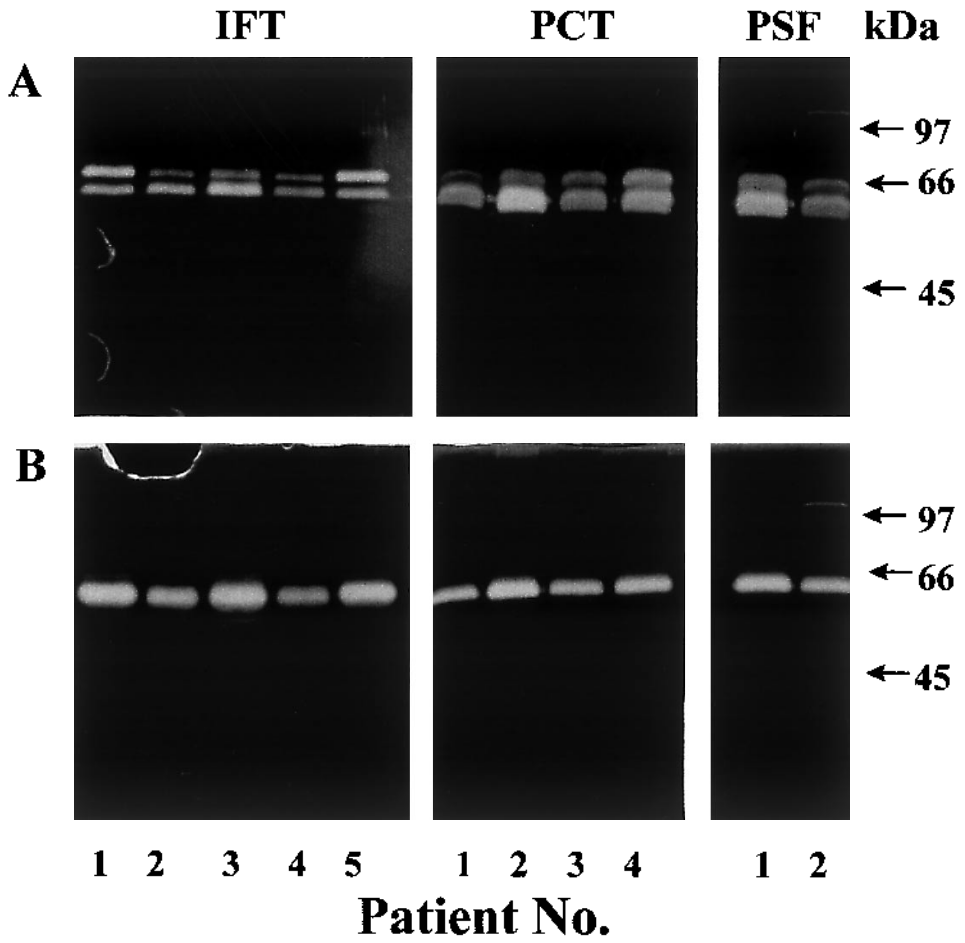

Figure 6. Gelatin zymography of the MMP-2 containing pool from Con A-Sepharose affinity chromatography, (A) before and (B) after treatment with APMA. IFT: interface tissue, PCT: pseudocapsular tissue, PSF: pseudosynovial fluid.

lysis band of 65 kDa appeared. At the position of the 67 kDa sharp band, a new more diffuse band of low intensity has migrated. The 92 kDa band corresponded to proMMP-9 and the 83 kDa to a preexisting, partially activated form of proMMP-9. Upon treatment with APMA, the major part of proMMP-9 was converted to 83 kDa and only a very low amount of it to the 67 kDa form. The initial 67 kDa sharp band may represent a contamination of proMMP-2, which after treatment with APMA was converted to its 65 kDa form, as described above (Figure 6). The observed conversion of proMMP-9 by APMA mainly to a stable partially activated form of 83 kDa strongly indicates that most of the proMMP-9 in all tissue samples and fluids existed in a complex form with TIMP-1. A TIMP-free proMMP-9 would be converted by this agent to a stable, fully activated form of 66 kDa[38,40,41].

DISCUSSION

Although it is reasonable to assume that cyclic mechanical loading combined with cellular host reaction to implants causes loosening of endoprostheses, the responsible molecular mechanisms are still unknown. In these processes the role of metalloproteinases is believed to be crucial. Takagi, *et al*[16], using the synthetic substrate DNP-S, reported that interface and pseudocapsular tissue extracts exhibited high collagenolytic and gelatinolytic activity, without necessitating preactivation by activating agents such as organomercurials. Since DNP-S is degraded identically by interstitial collagenases, gelatinases, and probably by the soluble form of MT1-MMP, which have been detected in periprosthetic tissues and fluid, the particular contribution of each enzyme in this activity cannot be evaluated.

Our study focused on evaluation of the contribution of several metalloproteinases in collagenolytic and gelatinolytic activity of periprosthetic tissues and fluid uninhibited by endogenous inhibitors, using the synthetic substrate DNP-S. Extraction of MMP from tissues in the presence of serine and cysteine proteinase inhibitors was performed to obtain extracts that more directly reflect the *in vivo* state. The extracted MMP were fractionated and studied separately. It

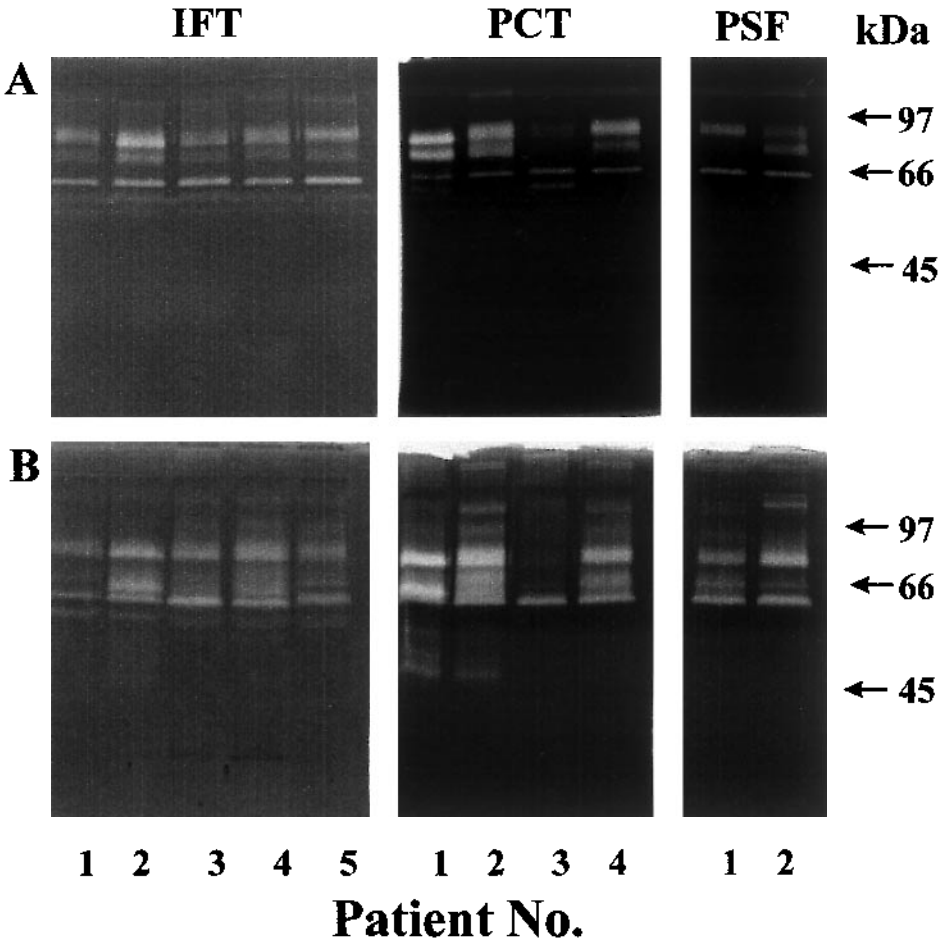

Figure 7. Gelatin zymography of the MMP-9-containing pool from Con A-Sepharose affinity chromatography, (A) before and (B) after treatment with APMA. IFT: interface tissue, PCT: pseudocapsular tissue, PSF: pseudosynovial fluid.

was observed that collagenases are exclusively responsible for DNP-S-degrading activity present in periprosthetic tissue extracts and fluids, while the gelatinases make no contribution. These collagenases are probably the MMP-13 and MMP-1, since they were detected in samples tested and since it is known that the MMP-8 exist in very low levels in periprosthetic tissues and fluid[17].

The presence of MMP-13 in the pseudosynovial fluid has been reported[44]. Our finding that the MMP-13 is also present in the extracts of periprosthetic tissues supports the suggestion[44] that this enzyme after its synthesis is also secreted into the extracellular milieu. The proenzyme of MMP-13 is not detected, perhaps because of low levels in samples. Low level of MMP-13 proenzyme compared to that of its activated form has been observed in pseudosynovial fluid[44]. The MMP-1 from these 2 collagenases is mainly found in its proenzyme form, while MMP-13 is in its activated form. Thus, it is reasonable to assume that the contribution of MMP-13 in DNP-S-degrading activity is more significant than that of MMP-1. Whether the observed immunoreactive bands of molecular mass 38 and 34.5 kDa correspond to activated species of MMP-13 is not known.

Yamanaka, *et al*[54] applied an extraction procedure, including homogenization of rheumatoid arthritis synovial tissue in the presence of the detergent Brij 35, followed by centrifugation at 10,000 g, to identify the presence of membrane-bound MT1-MMP in this tissue. Thus, the latent and active forms of MT1-MMP, 66 and 60 kDa, respectively, that were identified correspond to the membrane-bound enzyme form that is soluble by the detergent.

In our work the periprosthetic tissues were extracted in the absence of a detergent and the extracts were subsequently centrifuged at 100,000 g, thus cells and cell membrane particles that contain the membrane-bound MT1-MMP were removed in the precipitate. Only a soluble form of MT1-MMP would be expected to exist in the 100,000 g supernatant, while the pseudosynovial fluid samples, since they were not ultracentrifuged, would be expected to contain the membrane-bound MT1-MMP as well as its soluble form. Thus the detected immunoreactive 70/69 kDa duplex band

may represent the latent form of membrane-bound MT1-MMP, while the 56.5 kDa band represents a proteolytically processed soluble form of this metalloproteinase, as reported[43]. Thus, the contribution of a soluble form of MT1-MMP in DNP-S-degrading activity may be excluded, since this form was not detected in most samples tested. However, given that the MT1-MMP is expressed in periprosthetic tissues[42], its membrane-bound form may be involved in other degrading or activating processes. Due to the limited number of pseudosynovial fluid samples, we cannot explain why a soluble form of MT1-MMP was not detected in these samples, which is in contrast with data of other investigators[43].

The existence of both MMP-13 and MMP-1 activated species suggests that endogenous activators, which are present in periprosthetic tissues, activate the latent collagenase proenzymes *in situ*. The plasminogen activation system, which is upregulated in interface tissue[45], MMP-3, and MT1-MMP, which are present in interface tissue[17,20,42], may be responsible for the *in situ* activation of MMP-13 and MMP-1[26,31]. The exhibited DNP-S-degrading activity was uninhibited by endogenous inhibitors, indicating that an imbalance between the levels of these collagenases and TIMP could exist. This may be the result of the interplay of various cytokines and growth factors secreted by cells that accumulate in periprosthetic tissues, such as monocytes/macrophages, fibroblasts, and endothelial cells. Interleukin 1ß (IL-1ß), IL-6, IL-11, tumor necrosis factor-α, basic fibroblast growth factor, transforming growth factors TGF-ß 1 and 2, and platelet derived growth factor have been detected in periprosthetic tissues[8,19,23,24,55-58]. It is known that these factors, alone or in various combinations, have different effects on the production of MMP and TIMP by different cells; most of them upregulate the MMP production[25,59,60]. Recently, it was reported that expression of the extracellular matrix metalloproteinase inducer (EMMPRIN) is upregulated in interface tissue and that it is colocalized with MMP-1[61]. EMMPRIN can upregulate expression of several MMP, but has little effect on TIMP. The possibility that other factors in periprosthetic tissues that have not been yet identified may participate in the regulation of MMP and TIMP-1 levels cannot be excluded.

MMP-1 and MMP-13 may play an important role as initiators in connective tissue remodelling, since they degrade native type I and III collagens[29,31]. Further, MMP-13 degrades native type II collagen and gelatin as well as proteoglycans[31,62]. They may directly contribute to bone resorption by removing the osteoid layer from calcific bone, preparing the surface for osteoclasts to attach and begin resorption[63]. Thus, an imbalance of active MMP-1 and MMP-13 in relation to TIMP could be strongly implicated in bone resorption, since it is known that TIMP can inhibit *in vitro* stimulated bone resorption[64]. Moreover, activated MMP-13 and MMP-1 have the ability to activate the MMP-2[26], leading to enhancement of other proteolytic processes that may be involved in endoprosthesis loosening. Considering the above reports for MMP-1 and MMP-13 and that their activated species are present in periprosthetic tissues, it is assumed that these 2 collagenases play a key role in loosening of endoprostheses. Further studies with collagenase inhibiting drugs displaying different inhibitory effects against MMP-1 and MMP-13 may determine which of the 2 collagenases is mainly responsible for the local activity.

Periprosthetic tissues also contained high levels of both gelatinases. However, neither gelatinase A nor gelatinase B contributed to DNP-S degradation, because they existed mainly in complex with TIMP. It is known that the proenzymes of gelatinases in complex with TIMP can be activated by releasing the propeptide that covers their active site, in response to a number of activators. However, the TIMP present in the complex has the ability to recover the active site, resulting in inhibition of the activated proenzyme[39,40]. This observation raised the question of the role of gelatinases in periprosthetic tissues. It has been reported that neutrophil elastase can inactivate TIMP-1, which is present in the complex with proMMP-9, without significant destruction of proenzyme. Once TIMP-1 is inactivated, proMMP-9 can be readily activated by other activated MMP, such as MMP-3[65]. Elastase and MMP-3 have been detected in the interface tissue[17,20,46]. Thus, it is possible to speculate on *in situ* activation of proMMP-9/TIMP-1 complex by the synergistic effect of elastase and MMP-3. It is also known that MT1-MMP is implicated in the activation of proMMP-2 in complex with TIMP-2 through a process involving MMP-13[66-68]. Since these 2 MMP are present in periprosthetic tissues, *in situ* activation of proMMP-2/TIMP-2 complex may occur.

It is also known that TIMP in complex with gelatinase proenzymes not only prevent activation of the respective proenzyme, but they also can bind and inhibit other activated MMP. TIMP are bound to gelatinase proenzymes via their C-terminal, while their N-terminal, which is responsible for their inhibitory activity, remains free[37,39,40]. Thus, activated MMP-13 and MMP-1 may be partially masked by TIMP in complex with gelatinases, and the collagenase activity determined by DNP-S degradation corresponded only to free enzymes.

Although the number of cases in our study was limited, it should be noted that, independently of the type of prosthetic fixation or alloy and the presence or absence of focal osteolysis, both gelatinases were present in periprosthetic tissues in high levels, in agreement with other data[15]. It seems that induction of MMP-9 and elevated production of MMP-2 is a common feature of cellular host reaction occurring in periprosthetic tissues, leading to THA loosening. Thus, the precise role of gelatinases in the weakening of periprosthetic tissue remains to be elucidated.

A report on the collagenolytic and gelatinolytic potential

of periprosthetic tissues has shown that high DNP-S-degrading activity was also expressed in pseudocapsular tissue extracts, compared to interface tissue extracts[16], which concurs with our data. This observation, in association with the high content of gelatinases in pseudocapsular tissue, indicates that this tissue exists in an activated state resembling interface tissue. It is possible that pseudocapsular tissue may contribute to loosening of prostheses. Indeed, foreign body reaction may occur initially in pseudocapsular tissue and subsequently cells, cytokines, and enzymes originating from this tissue are released into pseudosynovial fluid and induce the formation of interface tissue between bone and implants. This hypothesis[16,69] is of particular interest, but remains to be clarified.

Our findings strongly indicate that periprosthetic tissues represent activated connective tissues involved in pathological matrix degradation, which may contribute to endoprosthesis loosening. The relevance of MMP to endoprosthesis loosening is of great therapeutic importance, because it is known that MMP can be pharmacologically inhibited. The use of such synthetic or recombinant inhibitors may modulate MMP activity, thus preventing the periprosthetic tissue weakening.

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