Several possible causes of aseptic loosening after total hip replacement (THR) have been proposed, among which wear-generated debris is generally accepted as the most important. It is probable that phagocytosis of wear particles by macrophages may initiate the release of a variety of soluble mediators, such as cytokines and free radicals, causing an aseptic inflammatory response which promotes the differentiation of bone-resorbing osteoclasts. Among these targets, several proteins involved in the differential protein expression in patients with aseptic loosening have been reported. However, the detailed molecular characteristics and pathogenic mechanisms for aseptic loosening are not fully understood.

High-level reactive oxygen species (ROS), such as superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH$^-$), are known to cause oxidative stress and damage to cells. Kinov et al showed that two oxidative stress markers, oxidized glutathione and malondialdehyde (MDA), were increased in peri-prosthetic tissues of patients with aseptic loosening compared with controls. Furthermore, endothelial cells grown in vitro on the surface of titanium alloy, showed signs of permanent oxidative stress and had less tolerance to H$_2$O$_2$ treatment than those grown as controls on polystyrene. Also, an increased level of intracellular ROS has been observed after cathodic polarisation of titanium alloy, which mimics corrosion. Notwithstanding this emerging evidence, the association between ROS and aseptic loosening needs to be addressed further.

Since the cellular responses would be different between patients with or without aseptic loosening, the aim of this study was to analyse the differential protein expression in patients with aseptic loosening of a THR and controls.

Patients and Methods

We began with a proteomic method to select the potential target proteins in the synovial fluids of patients with aseptic loosening. Among these targets, several proteins involved in the antioxidant or detoxification system were noticed. Extensive validation of the levels of the central ROS scavenging enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase, was also performed in patients with or without aseptic loosening after THR. The potential roles of these dysregulated ROS-related proteins (or enzymes) in the pathogenesis of aseptic loosening were considered.

Between February and December 2010, 41 patients were enrolled in the study and divided into three groups, namely primary THR (Group A), stable THR after revision for high polyethylene wear (Group B), and...
revision THR with aseptic loosening (Group C). The study protocol was approved by our Institutional Review Board and informed consent was obtained from each participant.

Group A comprised 16 patients who underwent primary THR for advanced osteoarthritis. There were six females and ten males with a mean age of 66.2 years (58 to 75). Specimens of synovial fluid and capsule were obtained at operation.

Group B comprised 10 patients who underwent revision THR for polyethylene wear. There were four females and six males with a mean age of 68.1 years (56 to 74). The mean interval between primary and revision THR was 16.0 years (10.2 to 19.3). Radiographs showed firmly fixed implants in all cases and this was confirmed at revision, when specimens of synovial fluid and capsule were obtained.

Group C comprised 15 patients with aseptic loosening after cementless THR. There were six females and nine males with a mean age of 64.2 years (52 to 78). The mean interval between primary and revision THR was 14.7 years (8.6 to 22.5). The loosening was confirmed radiologically and classified according to Paprosky, Perona and Lawrence\(^9\) as involvement of the cup, stem or both components. Septic hips were excluded from this group by analysis of inflammatory markers in the blood (C-reactive protein, ESR and leucocytosis) and by culture of synovial fluid.

Preparation of synovial fluid. About 5 ml of fluid was collected and mixed with 50 μl of protease inhibitor cocktail (Pierce Biotechnology Inc., Rockford, Illinois). The samples were centrifuged at 2500 rpm for 20 minutes at 4°C, and the supernatants then stored at -80°C until analysis. In order to prepare the samples for two-dimensional gel electrophoresis analysis, the synovial fluid was dialysed overnight at 4°C in distilled water to remove salts. The samples were then centrifuged at 75 000 rpm for three hours to remove the possible interference of DNA or RNA. The clear supernatant was desalinated further using two-dimensional (2-D) clean-up kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The concentrated samples were dissolved in rehydration buffer [7M urea, 2M thiourea, 4% 3-[3-cholomidopropyl]dimethylammoniol]-1-propanesulfonate (CHAPS), 0.4% dithiothreitol (DTT) and 0.001% bromophenol blue]. Protein concentration was measured using the Plus One 2-D Quant kit (GE Healthcare Bio-Sciences AB).

Two-dimensional gel electrophoresis (2-DE). The cell-free pooled synovial fluids from five patients in Group B and six from Group C were subjected to 2-DE. This was performed using Ettan IPGphor 3 and SE 600 Ruby (GE Healthcare Bio-Sciences AB). For isoelectric focusing, 100 μg protein was diluted with rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.4% DTT, and 0.001% bromophenol blue) to a total volume of 200 μl. Immobiline strips (pH 4 to 7, 11 cm; GE Healthcare Bio-Sciences AB) were rehydrated in this solution for 16 hours under mineral oil. Isoelectric focusing was performed at 20°C (GE Healthcare Bio-Sciences AB), with the following parameters: 200 V (one hour), 500 V (one hour), 1000 V (one hour), 5000 V (two hours) and 8000 V (approximately four hours). The current was limited to 50 μA per strip. After isoelectric focusing the immobilised pH gradient strips were stored at -80°C until analysis by sodium dodecyl sulfate-polyacrylamid gel electrophoresis (SDS-PAGE).

The immobilized pH gradient strips were then incubated in the equilibrium buffer (2% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl pH 8.8, 6M urea, 30% glycerol, 0.001% bromophenol blue and 1% DTT) for 15 minutes and followed by the same equilibrium buffer, except that DTT was replaced by 4% iodoacetamide, for 30 minutes at room temperature. The immobilized pH gradient strips were washed in SDS electrophoresis buffer (25 mM Tris base, 192 mM glycine, and 0.1% SDS), placed on top of 12.5% SDS-PAGE and sealed in place with a solution of 0.5% low-melting agarose in SDS electrophoresis buffer.

The electrophoresis current was 10 mA/gel for 20 minutes, followed by 20 mA/gel for 5.5 hours at room temperature. Silver staining. After electrophoresis, the gels were fixed in a mixture of 10% acetic acid and 40% methanol, sensitised in a mixture of 0.2% sodium thiosulphate, 6.8% sodium acetate and 30% ethanol, rinsed with de-ionised water, then silver-stained in 0.25% silver nitrate and 0.04% formaldehyde. The protein spots in the gels were developed in 2.5% sodium carbonate and 0.02% formaldehyde. The reaction was stopped by adding a solution of 50% methanol and 12% acetic acid.

Gel imaging. The stained polyacrylamide gels were scanned using an Amersham Image Scanner (GE Healthcare Bio-Sciences AB). The images and spot patterns were matched and analysed using Bio-Rad PDQuest 2-D Analysis Software Version 8.01 (Bio-Rad Laboratories Inc., Hercules, California).

Matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry. The spots of interest were excised, washed with de-ionised water, destained with 1% K\(_3\)Fe(CN)\(_6\) and 2.48% Na\(_2\)S\(_2\)O\(_3\), rehydrated with 100% acetonitrile, reduced with 50 mM NaHCO\(_3\) in acetonitrile, then digested with trypsin (2.5 ng/μl trypsin; Promega, Madison, Wisconsin) at 37°C for 16 hours. The digested products were extracted with 5% trifluoroacetic acid in 50% acetonitrile. Aliquots of these products were loaded onto AnchorChip followed by MALDI-TOF assessment using an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonik GmbH, Leipzig, Germany). The peptide mass data were analysed using Mascot search (Matrix Science, Boston, Massachusetts). The matched peptides having a MASCOT score over 65 and coverage over 20% of the protein were considered as candidates.

Western blot analysis. The prepared synovial fluids from all 41 patients were subjected to Western blot analysis and immunohistochemistry to examine the relative expression of the selected target proteins. Twenty-five micrograms of total proteins from the synovial fluids were mixed with...
Laemmli buffer containing 0.25M Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue and separated by 12% SDS-PAGE, along with a broad-range prestained protein molecular standard (Bio-Rad Laboratories Inc.). The proteins were electrotransferred (Bio-Rad Laboratories Inc.) onto polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories Inc.) which were blocked in 5% non-fat milk and incubated with diluted primary antibodies for two hours at room temperature or 4°C overnight. The primary antibodies used in the study were SOD1 (1:2000; Abcam, Cambridge, United Kingdom), SOD2 (1:2000), SOD3 (1:1000; Abcam), transthyretin (1:500; Millipore, Bedford, Massachusetts), serotransferrin (1:1000; Abcam), MMP-2 (1:1000; Abcam), catalase (1:2000; Abcam), peroxiredoxin (PRDX2) (1:2000; Millipore), GPx1 (1:400; Abcam), GPx2 (1:2000; Abcam) and immunoglobulin G (1:5000; Chemicon, Temecula, California). The membranes were then washed with the buffer containing 1x phosphate buffer saline (PBS) and 0.1% Tween 20 and incubated with secondary antibody, either anti-Rabbit immunoglobulin G antibody conjugated with horseradish peroxidase (1:5000; Chemicon) or anti-mouse immunoglobulin G antibody conjugated with horseradish peroxidase (1:5000; Chemicon). After extensive washing, the membranes were incubated with the enhanced chemiluminescence (ECL) substrates (Pierce Biotechnology Inc.) and exposed to X-ray film.

**Immunohistochemistry.** Since the histological sections from the osteolytic hip capsules contained granulomatous lesions with caseating necrosis, these materials were easily detached from the slides during immunostaining. Therefore, only about half of the capsular samples were shown to have successful immunohistochemistry staining. They were collected intra-operatively during primary or revision THR, fixed in 10% neutral buffered formalin for 12 hours at room temperature, embedded in paraffin and sectioned. The tissue sections were deparaffinised and rehydrated and, in order to increase immunostaining intensity, heated at 95°C for ten minutes in citrate buffer (0.01 M, pH 6). The sections were incubated with the primary antibody overnight at 4°C. The primary antibodies used in the immunohistochemistry experiments were SOD1, SOD2, SOD3, serotransferrin, catalase, PRDX2 and GPx1. Anti-mouse or anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase (Chemicon) was used as the secondary antibody (1:2000). After incubation, the tissue sections were washed three times for three minutes in PBS. The immunoreactive signals were seen using DAB (3,3-Diaminobenzidine) substrate which stained the target protein yellow. Three sections were examined for each hip capsule sample. The intensity of immuno-signals was evaluated as described by Denkert et al. whereby for each histological section, the staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong).

**Statistical analysis.** The differential expression of PRDX2, TTR, serotransferrin, and the ROS-associated enzymes (catalase, GPx1, GPx2, SOD1, SOD2 and SOD3) between patient groups were analysed using the Kruskal-Wallis test. Statistical analysis of individual proteins in immunohistochemical staining was also performed using the Kruskal-Wallis test. The gender, age and interval between primary and revision THR data were analysed by the chi-squared test. A p-value < 0.05 was considered to be significant.

**Results**

There was no statistical difference in gender and age among the groups (gender, p = 0.869 by chi-squared test; age, p = 0.237 by ANOVA analysis) and no significant difference in the interval from primary to revision THR between Groups B and C (p = 0.338 by t-test, Table I).
Initially, a comparative 2-DE was used to determine the potential differences of the global protein expression in synovial fluids between Groups B and C (Fig. 1). The cell-free synovial fluids pooled from five patients in Group B and six in Group C were subjected to 2-D electrophoretic gel analysis. Approximately 400 spots were observed on each 2-D gel. When the samples from the two groups were compared, those with a ≥ 5-fold increase or ≥ 5-fold decrease in Group C were selected for identification by mass spectrometry. Six of the 14 selective spots received reliable protein identification by MALDI-TOF mass spectrometry (Fig. 1, Table II). All six spot samples displayed a credible peptide score (> 65) and high coverage (> 20%) of the matched peptide to the target protein (Table II). Among these targets, diverse forms of serum albumin might be present at different abundant levels in the synovial fluid between Group B and C (Fig. 1; spots 4, 5 and 6). In addition to serum albumin, three spots with ≥ 5-fold increase in Group C were characterised as transthyretin, peroxiredoxin 2 (PRDX2) and serotransferrin (Fig. 1; spots 13, 7 and 11). Detailed information about these identified proteins is shown in Table II.

In order to validate the levels of transthyretin, PRDX2, serotransferrin, and the ROS-associated enzymes, Western blot analysis was performed to compare their levels in all synovial fluid samples from Groups A, B and C. Due to the potential association between elevated ROS and loosening of THR, we also determined the levels of the major ROS scavenging enzymes in synovial fluid samples. These enzymes included catalase, SOD1, SOD2, SOD3, GPx1, and GPx2. The representative Western blots using a fixed amount (25 μg) of total proteins are shown in Figure 2. Compared with Groups A and B, the levels of transthyretin and SOD3 in Group C were nearly three times greater. Conversely, GPx2 in Group C patients was decreased by up to four-fold compared with Groups A and B. In particular, patients with hip implants (Groups B and C) showed a lower amount of serotransferrin in hip synovial fluids than the controls (Group A). Although PRDX2 showed an up-regulated pattern in Group C patients, this
was not statistically different compared with Groups A and B (Fig. 2). Also there was no significant difference in Catalase, SOD1 and SOD2 between all three groups. It is of note that, whereas PRDX2, catalase, SOD1 and SOD2 are known as intracellular proteins, two extracellular proteins, MMP-2 and Immunoglobulin G (IgG), included in this study did not show obvious differences between the three groups. The statistically summarised data of the individual proteins in synovial fluid from different Groups is shown in Figure 3.

Immunohistochemical staining was used to evaluate the expression of the ROS-related proteins in capsular tissues. Since the sections contained granulomatous lesions with caseating necrosis, only about half of the samples showed successful immunohistochemical staining. Six ROS scavenging enzymes were determined, including PRDX2, catalase, SOD1, SOD2, SOD3 and GPx1. Apart from SOD3, the other enzymes are intracellular proteins. The staining intensities of each protein in the capsular tissues were scored from 0 (no staining) to 3 (strong staining). The capsular tissues in Group C had a much higher expression of PRDX2 and SOD2 than those in Groups A and B (PRDX2: Group A vs Group C, p = 0.012, Group B vs Group C, p = 0.015; SOD2: Group A vs Group C, p = 0.011, Group B vs Group C, p = 0.012, all by Kruskal-Wallis test). The mean score was 2.6 (SD 0.7) for PRDX2 and 2.5 (SD 0.6) for
SOD2 (Fig. 4). However, the levels of catalase, GPx1, SOD1 and SOD3 showed no significant differences among the three groups (Fig. 5).

**Discussion**

Aseptic loosening remains the most common cause for revision of a THR.\(^1\text{1,12}\) The chronic oxidative stress in periprosthetic tissue cells is suggested as one of the causative factors.\(^4\) However, alterations in the expression of the ROS scavenging enzymes or their related proteins in aseptic loosening has previously been unclear. We demonstrated for the first time that specific antioxidant enzymes or related proteins were up- or down-regulated in patients with aseptic loosening (Group C) compared with the two
control groups (Table III). These dysregulated ROS-related proteins may play important roles in the pathogenesis or progression of THR failure.

By using a proteomic approach to identify the potential biomarkers of THR loosening, transthyretin was identified as one of the potential molecular signatures for aseptic loosening (Fig. 1, Table II). Transthyretin is a major protein in extracellular fluids, a carrier of thyroid hormones and retinol and is known to be associated with the amyloid diseases, including senile systemic amyloidosis, familial amyloid polyneuropathy and familial amyloid cardiomyopathy, in which insoluble transthyretin fibrils are deposited in either peripheral nerves or heart tissue.13 Although the true role of transthyretin in these diseases is unclear, there is evidence that the circulating transthyretin in extracellular fluids functions to remove toxic or foreign substances. Recent findings have shown that transthyretin is a potent scavenger of beta-amyloid deposits and is

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**Fig. 4a**
Immunohistochemical staining and bar charts showing the expression of a) PRDX2 and b) SOD2 in hip capsule tissues from Groups A, B and C. Patients in Group C had a much higher expression of PRDX2 and SOD2 than those in Groups A and B (* PRDX2: Group A vs Group C, p = 0.012, Group B vs Group C, p = 0.015; SOD2: Group A vs Group C, p = 0.011, Group B vs Group C, p = 0.012 by Kruskal-Wallis test). The arrows represent the positive staining for the indicated proteins in capsular cells (× 200 magnification).

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**Fig. 4b**

involved in binding to many foreign aromatic compounds.\textsuperscript{14,15} In our study, we found that transthyretin was more abundant in synovial fluid of patients with aseptic loosening than in the controls (Figs 2 and 3). As in the diseases described above, the accumulation of transthyretin in the synovial fluid of the hip may be involved in the pathogenesis or progression of THR failure.

The down-regulation of serotransferrin in hip synovial fluids was verified in patients with a THR (Groups B and C) compared with those without one (Group A, Figs 2 and 3). Although the expression of serotransferrin at the initial 2-D gel screening was 5-fold higher in Group C than in Group B (Fig. 1, Table II), the validation test using all collected samples in the Western blot analysis showed only a 1.5-fold increase in Group C compared with Group B (Fig. 3). This discrepancy may be due to the smaller sample size in the initial 2-D gel analysis. Serotransferrin is an iron transport protein in blood plasma.\textsuperscript{16} The level of serotransferrin might be expected to be lower in patients with THRs (Groups B and C) than the controls (Group A), because excess iron may have been released from the implants.

Two major extracellular proteins involved in scavenging ROS are SOD3 and GPx2. The former is a catalyst for the

Table III. Summary of the dysregulation of the specific reactive oxygen species-related enzymes in hip synovial fluids and capsules. \textsuperscript{\textdagger} denotes no significant change versus Group A

<table>
<thead>
<tr>
<th>Location</th>
<th>Proteins*</th>
<th>Group A$^\dagger$</th>
<th>Group B</th>
<th>Group C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synovial fluid‡</td>
<td>TTR</td>
<td>-</td>
<td>-</td>
<td>Increased</td>
</tr>
<tr>
<td></td>
<td>SOD2</td>
<td>-</td>
<td>-</td>
<td>Increased</td>
</tr>
<tr>
<td></td>
<td>GPx2</td>
<td>-</td>
<td>-</td>
<td>Decreased</td>
</tr>
<tr>
<td></td>
<td>Serotransferrin</td>
<td>-</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>Capsules§</td>
<td>SOD2</td>
<td>-</td>
<td>-</td>
<td>Increased</td>
</tr>
<tr>
<td></td>
<td>PRDX2</td>
<td>-</td>
<td>-</td>
<td>Increased</td>
</tr>
</tbody>
</table>

\textsuperscript{*} TTR, transthyretin; SOD3, superoxide dismutase 3; GPx2, glutathione peroxidase 2; SOD2, superoxide dismutase 2; PRDX2, peroxiredoxin 2

\textsuperscript{\dagger} Group A used as an internal reference

\textsuperscript{‡} evaluated by Western blot analysis

\textsuperscript{§} evaluated by immunohistochemical staining
reduction of superoxide anions to hydrogen peroxide, while the latter reduces hydrogen peroxide to water by transferring the energy of the reactive peroxides to glutathione. 17,18 In the synovial fluid of patients with aseptic loosening (Group C), the level of SOD3 was significantly higher than in the other two groups. In contrast to SOD3, GPx2 was down-regulated in the Group C. The reason for this unbalanced activation of the antioxidant enzymes is unknown.

Five central intracellular enzymes acting against ROS damage, namely PRDX2, catalase, SOD1, SOD2 and GPx1, were studied in the capsular tissues. Of these, PRDX2 is a member of the peroxiredoxin family, which reduces hydrogen peroxide and alkyl hydroperoxides. 19 Similarly, catalase and GPx1 also act as catalysts with hydrogen peroxide in the formation of water and oxygen. Among these antioxidant enzymes, the up-regulation of PRDX2 and SOD2 was detected specifically in Group C. The other capsular enzymes did not show statistical differences among the three groups.

The involvement of ROS in the mechanism of aseptic loosening of THR has been suggested previously. 4 Inflammatory responses induced by wear debris in combination with cytokine release may gradually modulate the high-level production of ROS after THR. As a response to the toxic materials and ROS, the peri-prosthetic tissues may generate protective systems against these substances. High-level accumulation or expression of transthyretin, SOD3 and SOD2 may be responsible for the metabolic detoxification. However, in our study, not all of the antioxidant enzymes, especially catalase and GPx were equally over-expressed in patients with aseptic loosening. This inefficient expression of GPx (GPx2 in synovial fluid) or catalase may lead to the accumulation of H2O2. Moreover, there was a relatively low-level expression of serotransferrin in the synovial fluid of patients with THR (Fig. 3, Groups B and C), suggesting that high-levels of Fe2+/Fe3+ would be present in the surrounding cells. In the presence of Fe2+/Fe3+, the accumulated H2O2 may be converted into the far more reactive OH radical via the Fenton reaction 20 and thereby induce apoptosis in the affected cells by damaging lipid, protein and DNA. Based on our results, the dysregulated ROS-scavenging enzymes in combination with down-regulation of serotransferrin may contribute to the onset or progression of THR failure.

In conclusion, we found evidence that several ROS-related enzymes were expressed differentially in patients with primary osteoarthritis, and those with non-loosening and aseptic loosening of a THR. Despite the limitations of a small sample size, we suggest the possible mechanisms whereby these proteins may contribute to the pathogenesis and progression of THR failure.

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