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Expression of caspase-8 and caspase-3 proteins in interface membranes from aseptically loose total hip arthroplasty

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The terminal events leading to periprosthetic osteolysis are multifactorial and modulation of this process after the stage of mediator release has been demonstrated futile. We demonstrated that ceramic induce macrophage apoptosis *in vitro*. More recently, we and others demonstrated the presence of apoptosis in interface membranes (IMs) from aseptically loose total hip arthroplasty (THA). The purpose of this study was to characterize the mechanisms leading to apoptosis in these pseudomembranes. Western blot analysis was used to characterize the expression of caspase-3, caspase-8, poly(ADP-ribose)polymerase (PARP), and p53 proteins in IM from 35 patients (40 specimens) with a mean age of 58 years (range, 28–88 years) at the time of revision. Tissue harvested at the time of routine hardware removal served as control. Our results show that caspase-3 and caspase-8 pro-enzymes were expressed in both control and IM tissues. Our results also showed that caspase-3 active fragment (17 kDa) as well as caspase-8 active fragment (18 kDa) were expressed in IMs but absent in control tissues. We also demonstrated that both the native PARP (113 kDa) and its proteolytic fragment (89 kDa) were present in osteolytic IMs. Control membranes expressed only the 113 kDa native form of PARP. The over-expression of caspase-3 caspase-8 active fragments and the presence of PARP fragment were observed on both the acetabular and femoral sides of the prostheses. Finally, our results showed the absence of p53 expression in both osteolytic IMs and control tissues. In conclusion, our results suggest that the caspase/PARP pathway plays an active role in the activity of IMs from aseptically loose THAs.

Introduction

Aseptic loosening secondary to wear particle-induced osteolysis is the main cause of total hip arthroplasty (THA) failure. Phagocytosis of particulate debris by macrophages incites a cascade of events that results in the release of substances capable of stimulating bone resorption [1, 2]. The exact mechanisms by which the inflammatory cells interact with osteoblast/osteoclast cells and the relative importance of the various chemical mediators on ultimate bone lysis remain poorly understood.

From a general point of view, there are two distinct modes of cell death: necrosis and apoptosis. Necrosis is a passive form of cell death in which the cell responds to injury by rapid swelling, random uncontrolled DNA fragmentation, loss of cell membrane integrity, and leakage of cellular contents into the pericellular environment with the induction of an inflammatory response. Apoptosis or programmed cell death is an active form of cell death that is characterized by cell shrinkage, surface blebbing, organized fragmentation of DNA into 180 base pair units and release of apoptotic bodies which are eliminated by phagocytosis [3]. Apoptosis is a tidy and discrete mode of cell death where the elimination of apoptotic bodies is not associated with an inflammatory response.

The preferential induction of macrophage apoptosis in periprosthetic tissues would be a desirable therapeutic modality since little or no inflammatory response is generated. Indeed, some studies have shown that macrophage apoptosis can be induced *in vitro* with metal ions [4, 5] or ceramic particles [6, 7]. We [8] and others [9, 10] have also identified apoptotic macrophages in interface membranes harvested *in vivo* at the time of revision THA. The explosion of interest in apoptosis lies in the fact that being genetically regulated, it offers

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potential specific targets for therapeutic intervention [11]. However, the exact cellular mechanisms leading to apoptotic cell death in IMs remain unknown.

Many intracellular proteases, namely caspases, participate in the apoptotic process in a cascade analogous to the compliment and coagulation systems. Upstream caspases (e.g. caspase-8) cleave and activate downstream caspases (e.g. caspase-3), which in turn cleave the various substrate proteins that account for many of the biochemical and morphological changes that occur during apoptosis [12, 13]. Among the proteases in the effector pathway, caspase-3 is probably best correlated with apoptosis [14].

Activation of caspases during apoptosis results in the cleavage of critical cellular substrates [14, 15] including poly(ADP-ribose)polymerase (PARP), a nuclear protein implicated in DNA repair [16]. The proteolytic cleavage of PARP results in 89 and 24 kDa fragments [17]. The specific cleavage of PARP is another event that is closely associated with apoptosis [17, 18], p53 is another critical participant in a signal transduction pathway that mediates apoptosis in response to DNA damage by various treatments [19–21].

Since caspase-3 and p53 proteins are considered important effectors in apoptosis, the purpose of this study was to characterize their expression in interface membranes of aseptically loose THAs to improve our understanding of the mechanisms leading to periprosthetic osteolysis. Upstream (caspase-8 activation) and downstream (PARP cleavage) events related to caspase-3 activation were also evaluated.

Materials and methods

Materials

Novex 10% and 16% pre-cast SDS-PAGE gels (Tris-glycine) were purchased from Helixx Technologies (Scarborough, Ontario, Canada). Antibodies (anti-poly(ADP-ribose)polymerase, anti-caspase-3, anti-caspase-8, anti-p53) were purchased from Zymed Laboratories (InterMedico, Markham, Ontario, Canada). Peroxidase-conjugated goat anti-rabbit, goat anti-mouse, and goat anti-rat IgG were also bought from Zymed.

Specimen collection

Only cases revised for aseptic loosening were included in this study which has been approved by the Research and Ethics Committee of the hospital. Specimens of interface membranes (IMs) from 35 patients ranging in age from 28 to 88 years old (mean, 58 years) were harvested at the time of revision surgery. The duration of implantation ranged from 10 to 25 years (mean, 14 years).

Control tissues were obtained from patients undergoing hardware removal. The ideal control tissue for the wear particle-induced inflammatory response that occurs around a loose THA does not exist. The authors feel that the pseudocapsule that forms around hardware implanted for fracture fixation serves as a suitable control, since it too represents a foreign body response without the release of chemical mediators of osteolysis. Five specimens of control tissue were obtained from removal of hip screws and plates and three (3) specimens were from around tension band wires.

Protein extraction and Western blot analysis

Western Blot analyzes were performed as we previously described [7]. Briefly, each specimen was washed in cold phosphate-buffered saline (PBS), pH 7.5, snap frozen immediately on dry ice, and stored at $-80\,^{\circ}C$ for later protein extraction. Pieces of frozen specimen were sectioned off (~ 50 mg) and homogenized in 250 µl of lysis buffer (50 mM Tris-HCl (pH 7.5) – 5 µl of protease inhibitor cocktail (Sigma-Aldrich Canada, Oakville, Ontario)/ml of buffer – 1% SDS – 2% β-mercaptoethanol). These cell extracts were spun at $5000 \times g$ for 15 min and the supernatants were collected as the source of total protein. Protein concentration was measured using the Bio-Rad protein assay with BSA as standard. Seventy-five micrograms of total proteins from each sample were denatured at $100\,^{\circ}C$, loaded onto 10% (PARP) or 16% (caspase-3, caspase-8 and p53) acrylamide gels and separated by SDS-PAGE. Ten microliters of recombinant human p53 protein (Zymed Laboratories) were used as positive control for p53 experiments. Proteins were transferred to a nitrocellulose membrane using Novex Tris-Glycine Transfer Buffer. Blottings were performed using anti-PARP, anti-caspase-3, anti-caspase-8, and anti-p53 diluted 1 : 1000 as primary antibodies with peroxidase-conjugated anti-rabbit (1 : 10 000 – PARP and caspase-3). peroxidase-conjugated anti-rat (1 : 10 000 – caspase-8), or peroxidase-conjugated anti-mouse (1 : 10 000 – p53) as secondary antibodies. NEN Renaissance luminescence reagents were used for detection.

Results

Fig. 1 shows the Western blot analysis of caspase-8 expression in IMs of failed THAs. Fig. 1(A) demonstrates that caspase-8 pro-enzyme (doublet at ~ 55 kDa) is expressed in both control and IM tissues. The active fragment (18 kDa) is expressed in 90% of IM specimens (36/40 specimens) and absent in control tissues. The incidence of expression of active caspase-8 was similar on both acetabular and femoral sides of prostheses (Fig. 1(B) and Fig. 5). Other already described caspase-8 degradation products were visible between 55 and 37 kDa in both control and IM samples. A non-specific band at 37 kDa, identified as a non-specific band by the manufacturer, was also detected in both control and IM specimens.

Fig. 2 shows the Western blot analysis of caspase-3 expression in IMs of failed THAs. Fig. 2(A) demonstrates that caspase-3 pro-enzyme (32 kDa) is expressed in both control and IM tissues. The active fragment (17 kDa) is expressed in 92% of IM specimens (37/40 specimens) and absent in control tissues. The incidence of expression of active caspase-3 was similar on both acetabular and femoral sides of prostheses (Fig. 2(B) and Fig. 5).

Fig. 3 shows the Western blot analysis of PARP expression in IMs of failed THAs. Fig. 3(A) demonstrates the presence of the native form of PARP (113 kDa) in both control and IM tissues. The proteolytic fragment of PARP (89 kDa) is expressed in 95% of IMs

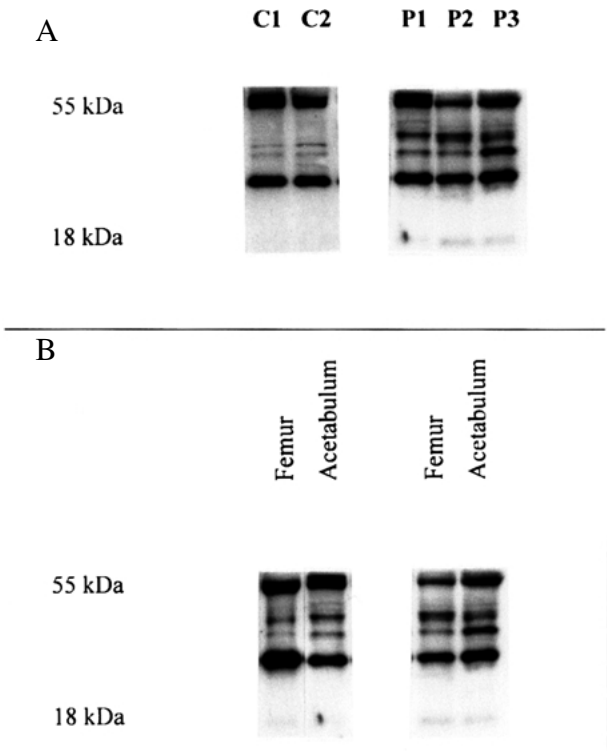

Figure 1 Expression of caspase-8 in interface membranes from aseptically loose THAs. Proteins from control (C1–C2) and interface membrane (P1–P2–P3) tissues were extracted and analyzed on 16% acrylamide gels (A). Tissues from the acetabular and femoral sides were compared (B). The percentage of tissues that expressed the active caspase-8 fragment is summarized in Fig. 5.

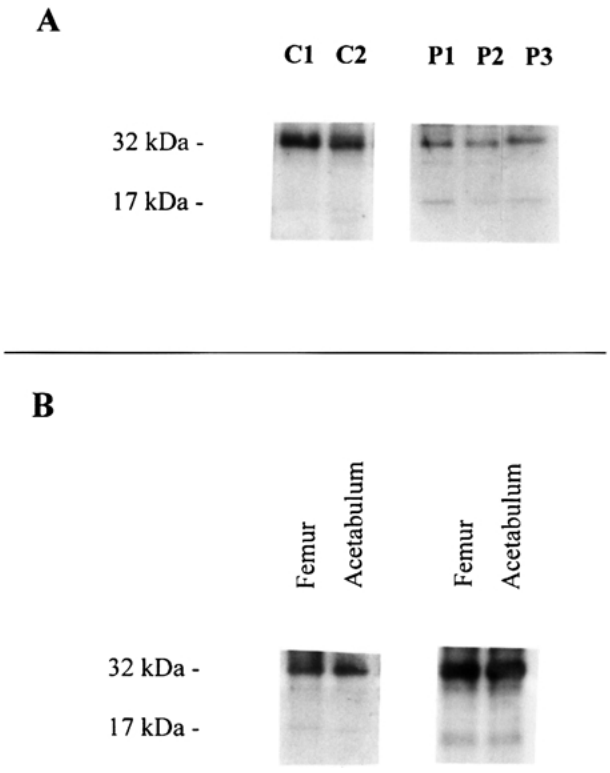

Figure 2 Expression of caspase-3 in interface membranes from aseptically loose THAs. Proteins from control (C1–C2) and interface membrane (P1–P2–P3) tissues were extracted and analyzed on 16% acrylamide gels (A). Tissues from the acetabular and femoral sides were compared (B). The percentage of tissues that expressed the active caspase-3 fragment is summarized in Fig. 5.

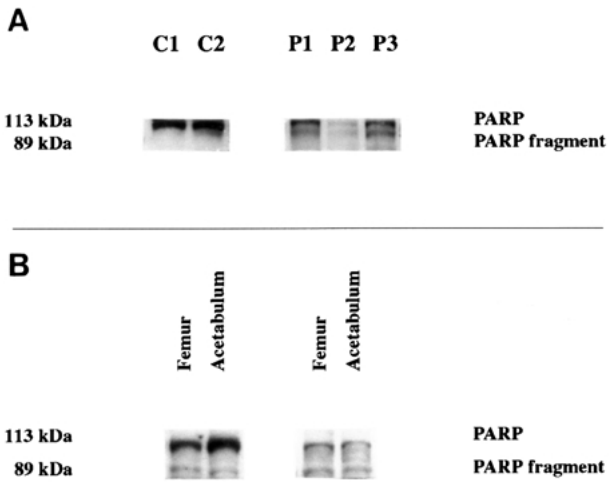

Figure 3 Expression of PARP in interface membranes from aseptically loose THAs. Proteins from control (C1–C2) and interface membrane (P1–P2–P3) tissues were extracted and analyzed on 8% acrylamide gels (A). Tissues from the acetabular and femoral sides were compared (B). The percentage of tissues that expressed the proteolytic fragment of PARP is summarized in Fig. 5.

we studied (38/40 specimens) and absent in control tissues (Fig. 3(A)). The incidence of the 89 kDa PARP fragment was similar on both acetabular and femur sides of prostheses (Fig. 3(B) and Fig. 5).

We were unable to detect p53 band in both control and IM tissues even after 2 h film exposure while recombinant p53 protein was detected after 30 s exposure time in the same experimental conditions (Fig. 4).

Fig. 5 summarizes our results on the expression of caspase-8, caspase-3, PARP, and p53 proteins in IMs from failed THAs. Results clearly show the activation of the caspase/PARP pathway in IMs from failed THAs whereas p53 induction is not expressed in control and IM tissues.

Discussion

Our results suggest that a caspase pathway is implicated in the induction of apoptosis in IMs from aseptically

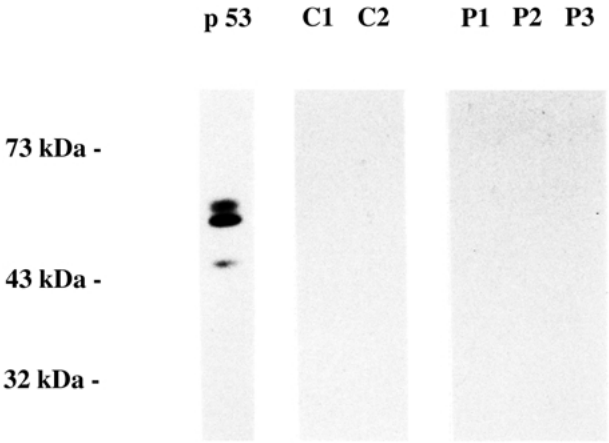

Figure 4 Expression of p53 in interface membranes from aseptically loose THAs. Proteins from control (C1–C2) and interface membrane (P1–P2–P3) tissues were extracted and analyzed on 16% acrylamide gels. Recombinant p53 was used as a positive control. The percentage of tissues that expressed p53 is summarized in Fig. 5.

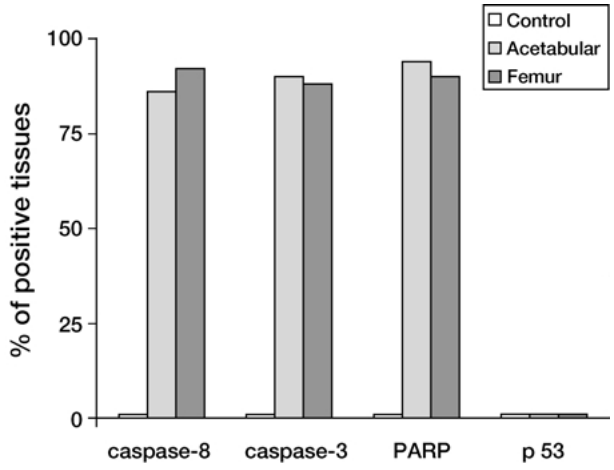

Figure 5 Protein expression in interface membranes from aseptically loose THAs. The figure summarizes the expression of caspase-3, caspase-8, PARP, and p53 in PMs. Results are expressed as the percentage of tissues showing the expression of caspase-3 and caspase-8 active fragments, proteolytic PARP fragment and p53. Results are from 30 tissues from 25 patients and are presented in light of their acetabular or femoral origin.

loose THAs. More specifically, caspase-3 and -8 are activated in these tissues. Caspases are synthesized as relatively inactive zymogens (pro-enzymes) that become activated by scaffold-mediated transactivation or by cleavage via upstream proteases in an intracellular cascade [22]. Caspase-8 represents one of the most receptor-proximal caspase in the Fas/TNF pathway and its activation can induce a cascade of caspases including caspase-3 [23, 24]. Because of its localization at or near the membrane, it has been suggested that this enzyme initiates this death pathway [25, 26]. Our results suggest that caspase-8 can be the initiating protease of the apoptotic pathway in IMs. The stimulation of a death receptor (CD95/Fas/TNF/APO-1) is not absolutely necessary for the activation of caspase-8 and oligomerization at the membrane seems to be sufficient for caspase-8 auto-activation [27]. The caspase cascade can therefore be ignited by intra- or extra-cellular signals but our results do not allow us to discriminate the exact biological activating factor. However, recent results showed that metal particles correlated with the presence of apoptosis in peri-implant tissues [10], suggesting that wear debris are external non biological inducers of apoptosis in these tissues. In this regard, some studies reported the *in vitro* stimulation of macrophage apoptosis by corrosion products of metal particles, namely metal ions [4–5], and ceramic particles [6–7].

At the biological level, TNF-α appears as a potential extra-cellular factor responsible for caspase activation in IMs from failed THAs. Indeed, TNF-α is a bone-resorbing mediator that has been detected at the bone-prosthesis interface [28–30] and is produced by macrophages in response to orthopaedic wear particles [30–35]. It is therefore possible that TNF-α acts on the periprosthetic environment to initiate cell death in an autocrine or paracrine manner by binding to its membrane receptor known to induce the caspase-8 pathway [36, 37]. Matrix metalloproteinases, a family of homologous enzymes playing a central role in the breakdown of extracellular matrix [38, 39], are also produced by IMs from failed THAs [40] and data suggest that matrix metalloproteinases and tissue inhibitors of proteinase systems participate in tissue remodeling in hip replacement and may contribute to implant loosening and osteolysis around implants [40]. Indeed, these proteins participate in many normal processes, including bone remodeling. These proteins are also known to induce apoptosis through the activation of Fas [41], a member of the TNF family also known to induce caspase-8 [42].

The bcl-2 family of proteins (at least 15 proteins) also plays an important role in the control of apoptosis. Some members of this family, such as bcl-2, function as cell-death suppressors and other homologues, including bax, bad, and bak have powerful death promoting abilities. Although the molecular mechanisms by which these proteins exert their pro- and anti-apoptotic functions remain poorly defined, it is well known that they play a pivotal role in cells undergoing apoptosis by interfering with the caspase family of proteins [43–44]. With regard to periprosthetic osteolysis, the *in situ* analysis of interface membranes of aseptically loosened orthopaedic implants showed that bax is expressed mainly in macrophages. However, not all cells that express this apoptotic-promoting protein enter into the phase of cell death [9]. These results suggest that caspase activation is not the only mechanism implicated in the induction of apoptosis in interface membrane from loosed implants. The characterization of the expression of both caspase and bcl-2 family of proteins *in vitro* is therefore of interest for the understanding the effect of orthopaedic particulate debris on macrophages.

In conclusion, our results suggest that a caspase/PARP pathway is activated in IMs from failed THAs. However, further studies are necessary to understand the initiation of this caspase pathway. Moreover, the study of other substrates of caspase-3 (e.g. U1-70 kDa) and the expression, both *in vivo* and *in vitro*, of the bcl-2 family of proteins known to interact with caspase activation appear of crucial interest for the understanding the caspase activity in IMs of aseptically loose THAs.

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