Apoptosis in interface membranes of aseptically loose total hip arthroplasty

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The terminal events leading to periprosthetic osteolysis are multifactorial in nature and modulation of this process after the stage of osteolytic mediator release has been futile. Recently, the demonstration of the ability of bisphosphonates to inhibit bone resorption that is mediated by particle-stimulated macrophages and their induction of osteoclast apoptosis suggests a potent area for modulation of osteolysis at the prosthesis-bone interface. The purpose of this study was to determine the mode of cell death that occurs at the osteolytic interface of failed total hip arthroplasty (THA). TUNEL staining, DNA laddering, and immunodetection of poly(ADP-ribose)polymerase (PARP) protein were used to identify the presence of apoptosis in interface membranes from 25 patients aged 28–88 years old (mean, 58 years) harvested at the time of hip revision surgery. Our results demonstrated positive TUNEL stain in 100% of specimens with an average 37% of cells (range 12–60%) positively stained for TUNEL whereas less than 8% of control tissue cells showed positive staining. DNA laddering, a characteristic feature of apoptotic cells, was observed in 82% (28/34) of specimens studied at both the acetabular and femoral side of aseptically loose THAs. No laddering was observed in control tissues. Finally, using Western blot analysis, we observed the appearance of the 89 kDa PARP fragment associated with apoptosis in 92% of specimens (30/33). Our results demonstrate the presence of apoptotic cell death in interface membranes of THAs suggesting that apoptosis-related events are indeed associated with periprosthetic osteolysis and could serve as a specific target point for therapeutic modulation.

1. Introduction

Loosening and osteolysis secondary to prosthetic wear debris are the most important processes limiting the longevity of total hip arthroplasty (THA). Osteolytic lesions result from a foreign-body inflammatory response to particulate debris [1], manifested by the appearance of a proliferating interface membrane which leads to prosthetic loosening [2, 3]. The macrophage, which represents 60–80% of the cell population of interface membrane harvested at revision surgery [4], is the key cell responsible for many of the events associated with osteolysis. Phagocytosis of particulate debris by macrophages incites a cascade of events that results in the release of substances capable of stimulating bone resorption. These substances include IL-1, TNF-α, and PGE_2 [5–9]. 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.

Recently, the use of bisphosphonates (BP) has shown some potential in preventing periprosthetic osteolysis. BPs are a class of synthetic compounds that are powerful inhibitors of bone resorption both *in vitro* [10] and *in vivo* [11]. They are well tolerated and effective in the treatment of various metabolic bone diseases [12, 13]. Horowitz and Gonzalez [14] and Sabokbar *et al*. [15, 16] have inhibited bone resorption using pamidronate and etidronate respectively in macrophage/bone co-culture models. Shanbhag *et al*. [17] were successful in inhibiting osteolysis in a canine model of periprosthetic osteolysis by administering oral alendronate (Fosamax). One of the mechanisms of action of the bisphosphonates on bone resorption, both *in vivo* and *in vitro*, is the induction of osteoclast apoptosis [18].

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

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the pericellular environment with the induction of an inflammatory response. Apoptosis, or programmed cell death, is an active form of cell death that is under positive and negative genetic control [19]. Apoptosis is characterized by cell shrinkage, surface blebbing, organized fragmentation of DNA into 180 base pair units and release of apoptotic bodies, which are then phagocytosed by neighboring phagocytes [20]. Apoptosis is a tidy and discrete mode of cell death where the entire process remains within the confines of an intact cellular membrane. Both necrosis and apoptosis can occur in response to a toxic stimulus. However, the physiologic implications of necrosis and apoptosis are distinctly different [21]. Necrosis is associated with an inflammatory response and tissue injury, whereas in apoptosis, the elimination of apoptotic bodies is not associated with an acute inflammatory response.

Cell death by apoptosis has been identified in many organ systems and is either detrimental or desirable [22]. For instance, excess apoptosis is detrimental in numerous autoimmune disorders and neural degenerative diseases such as Alzheimer's. The induction of apoptosis is, on the other hand, desirable in embryonic organ regression and in the elimination of cells with genetic mutations that can lead to cancer. The preferential induction of macrophage apoptosis in periprosthetic tissues would be a desirable therapeutic modality since little or no inflammatory response is generated. Indeed, Granchi *et al*. and Catelas *et al*. have shown that macrophage apoptosis can be induced *in vitro* with metal ions [23] or ceramic and polyethylene particles [24].

While it is known that the interface membrane (IM) induced by wear particles is laden with necrotic cells [25], to the best of our knowledge, it is unknown whether apoptotic cells are present in these tissues. The purpose of this study was to determine if apoptotic cell death occurs in interface membranes of failed THAs.

2. Materials and methods

2.1. Materials

Phenol/chloroform/isoamyl alcohol (25 : 24 : 1), chloroform/isoamyl alcohol (24 : 1), sodium acetate, anhydrous ethanol (denatured), and RNAse A (pancreatic) were purchased from Amresco (Solon, OH). Proteinase K was bought from Canadian Life Technologies (Burlington, Ontario, Canada). Novex 10% pre-cast SDS-PAGE gels (Tris-glycine) were purchased from Helixx Technologies (Scarborough, Ontario, Canada). Agarose was from ICN Pharmaceuticals (Montreal, Quebec, Canada). The anti-poly(ADP-ribose)polymerase (PARP) antibody was purchased from Boehringer Mannheim (Laval, Québec, Canada). Peroxidase-conjugated AffiPure Goat Anti-rabbit IgG were bought from Jackson ImmunoResearch (BIO/CAN Scientific, Mississauga, Ontario, Canada).

2.2. Specimen collection

Only cases revised for aseptic loosening of total hip arthroplasty were included in this study which has been approved by the Research and Ethics Committee of the Sir Mortimer B. Davis – Jewish General Hospital. All cases revised for infection were excluded. Preoperative consent was obtained from each patient. At time of surgery, specimens of interface membranes from 25 patients ranging from 28 to 88 years old (mean, 58 years) were harvested at the revision site. The duration of the implantation before revision surgery ranged from 5 to 21 years (mean, 13 years). All revised THAs had a metal-on-polyethylene articulating interface. There were 12 cemented, 2 hybrid, and 11 cementless THAs revised.

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 cases of control tissue were obtained from removal of hip screws and plates and three cases were from around tension band wires.

2.3. Tissue processing

Each specimen was washed in cold phosphate-buffered saline (PBS) and divided into two halves. The first portion was fixed in 10% buffered formalin for 3–5 h and saved for histological studies. Specimens were then dehydrated and embedded in paraffin. Two to five micron-thick serial sections were cut and placed on slides. The sections were stored at $-20\,^{\circ}C$ for future TUNEL stain analysis. The remaining half of the specimen harvested at revision surgery was snap frozen on dry ice and stored at $-80\,^{\circ}C$ for later DNA and protein extraction.

2.4. Nuclear labeling – DNA fragmentation analysis (TUNEL stain)

DNA fragmentation, a characteristic feature of apoptosis, was determined using *in situ* labeling of apoptosis-induced strand breaks [26]. In this analysis, terminal deoxynucleotidyl transferase (TdT) was used for the incorporation of biotinylated fluorescein labeled nucleotide (dUTP) to the 3′ end of fragmented DNA *in situ*. Slides were deparafinized using Hemo-De (Fisher Scientific, Montréal, Québec, Canada) and hydrated with graded ethanol concentrations from 100% to 50%. Samples were then analyzed by Hematoxylin and Eosin (H&E) staining (Shandon staining kit, Pittsburgh, PA) or digested with Proteinase K (20 µg/ml in 10 mM Tris-HCl, pH 7.4–8.0) for TUNEL stain. Slides were incubated 1 h at 37 °C with TUNEL reaction mixture, washed with Phosphate Buffered Saline (PBS), stained 5–10 min at room temperature with propidium iodide (PI) (0.2 µg/ml in PBS) that stained all nuclei, and washed again with PBS. Slides were mounted with Immu-Mount (Shandon, Pittsburgh, PA), analyzed under fluorescence microscopy, and pictures taken. The percentage of apoptotic cells per section of IM was determined by the ratio of TUNEL positive/PI positive cells [27]. Three microscopic fields of 75–200 cells (× 500 magnification) on three different sections (total of 9 fields) were analyzed for each specimen.

2.5. DNA extraction and agarose gel electrophoresis

One hundred milligram (100 mg) fragments were sectioned off the frozen specimens, homogenized in 500 µl of lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 0.5% SDS, and 10 mM EDTA at pH 8.0), and incubated at 37 °C for 30 min with 5 µg/µl RNAse A, and then 3 h at 50 °C with 100 µg/ml proteinase K to remove RNA and proteins from DNA preparations [28]. DNA was extracted twice with an equal volume of phenol/chloroform mixture and once with chloroform/isoamyl alcohol. Next, DNA was precipitated from the aqueous phase with two volumes of ethanol and 0.3 M sodium acetate, and stored overnight at −80 °C. DNA was recovered by centrifugation at 10 000 × *g* and the samples (10 µg) were loaded onto 1.5% agarose gel containing 50 µg/ml ethidium bromide, and run at 50 V for about 1 h 30 min. DNA was then visualized directly upon illumination with UV light and photographed using Polaroid type 667 film (ASA 3000).

2.6. Protein extraction and Western blot analysis

Pieces of frozen specimen were sectioned (∼ 50 mg) and homogenized in 150 µl of lysis buffer A (25 mM Tris–HCl (pH 8.0); 50 mM glucose; 10 mM EDTA; 1 mM phenylmethylsulfonylfluoride (PMSF) and 75 µl of lysis buffer B (50 mM Tris–HCl (pH 6.8); 6 M Urea; 3% SDS and 6% β-mercaptoethanol). These cell extracts were spun at 5000 × *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 (75 µg) of total proteins for each sample were denatured at 100 °C, loaded on a 10% gel and separated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane using Novex Tris-Glycine Transfer Buffer. Blotting was performed using anti-PARP diluted 1 : 1000 as primary antibodies with peroxidase-conjugated anti-rabbit (1 : 10 000) IgG as secondary antibodies. Boehringer luminescence substrate was used for detection. Autoradiographies were performed using Kodak X-Omat LS X-ray film.

2.7. Statistical analysis

The evaluations of the percentage of TUNEL stained cells were compared by the Student *t* test, while DNA laddering and protein analyses were compared using a comparison of proportions for independent samples (Chi-Square test). $P < 0.05$ was considered significant.

3. Results

3.1. Hematoxylin and Eosin (H&E) staining

Fig. 1 shows the H&E staining of both control (Fig. 1a) and IM (Fig. 1b) specimens. The control tissue in Fig. 1a shows the typical membrane that forms in response to fracture fixation hardware in the form of numerous fibrous cells in a bed of connective tissue collagen fibers. The particulate debris-generated interface membrane in Fig. 1b is typically characterized by a predominantly macrophage and giant cell infiltrate [4].

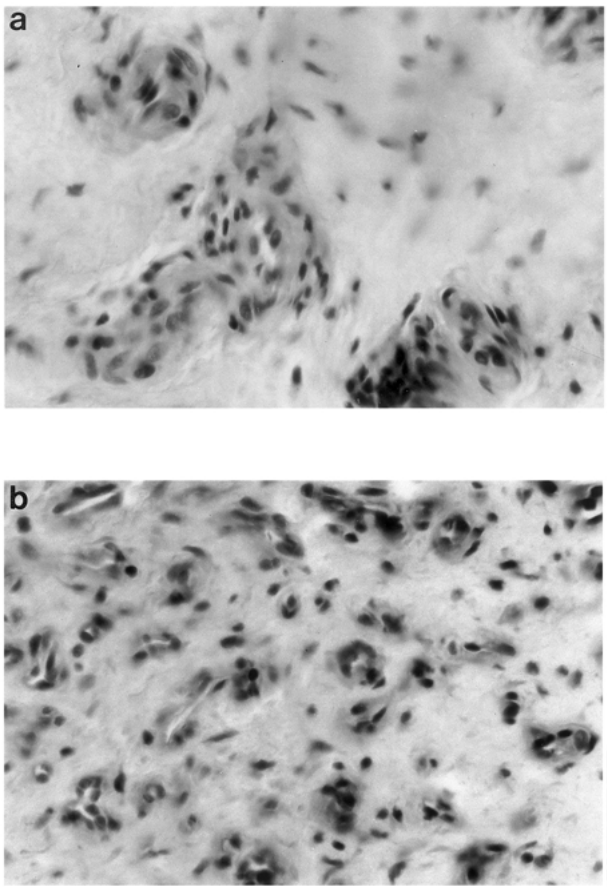


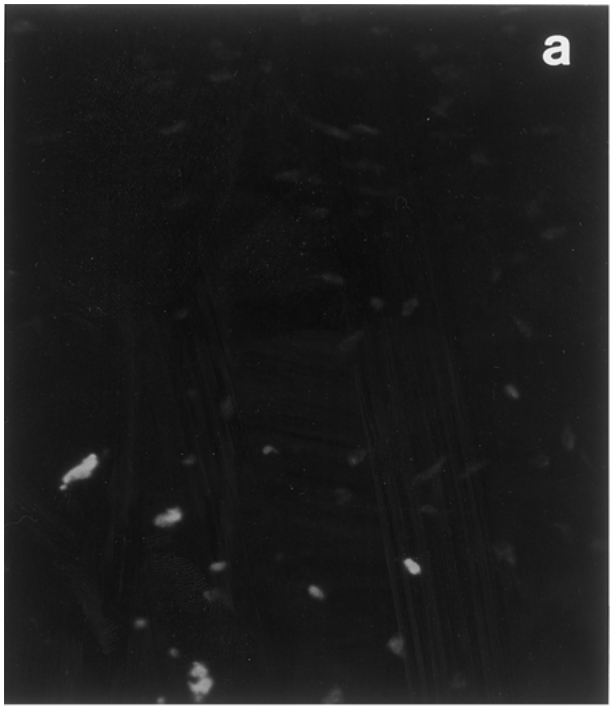
Figure 1 Hematoxylin and Eosin staining of control and interface membrane tissues. The control tissue in (a) shows the typical membrane that forms in response to fracture fixation hardware in the form of numerous fibrous cells in a bed of connective tissue collagen fibers. The particulate debris-generated interface membrane in (b) is characterized by a predominantly macrophage and giant cell infiltrate.

3.2. DNA fragmentation analysis

Fig. 2 shows the *in situ* labeling of apoptosis-induced strand breaks by TUNEL stain while Fig. 3 shows the results of DNA laddering in interface membranes of failed THAs. Our results demonstrated negative TUNEL stain for control tissues (Fig. 2a) (less than 10% staining was considered as negative). We observed positive TUNEL stain in 100% of interface membrane specimens (Fig. 2b). The range of cell staining was 12–60% (mean, 37%) for the 25 specimens we studied and was similar for tissue obtained from both acetabular and femoral sides of the revised prostheses (Fig. 5). We also observed the presence of DNA laddering in 82% of specimens (28 ladders/34 specimens) (Fig. 2). The presence of DNA laddering was observed with a similar incidence on acetabular and femoral sides of the prostheses (Fig. 5). No laddering was observed in control tissues.

3.3. Western blot analysis

Fig. 4 shows the Western blot analysis of PARP expression in interface membranes of failed THAs. Our results show the presence of both the 113 kDa native



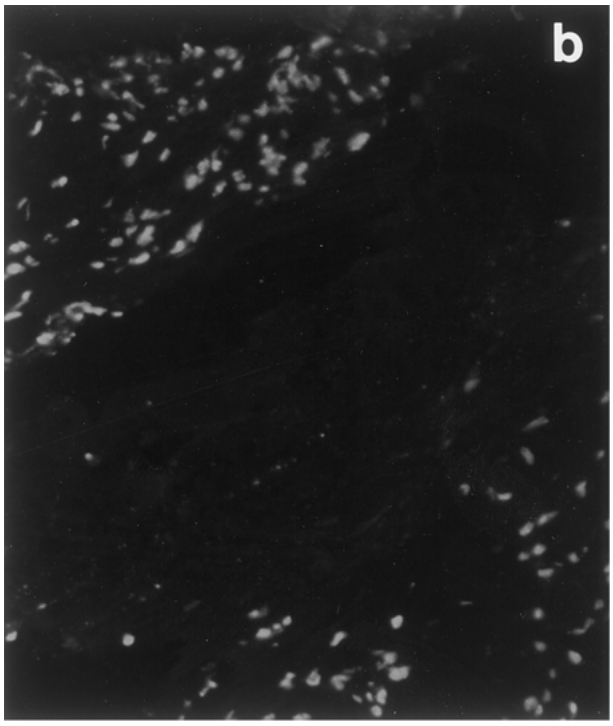


Figure 2 *In situ* labeling of apoptosis-induced strand breaks in interface membranes of failed THAs by TUNEL stain. Relatively few positively stained cells in control tissue (Fig. 2a) compared to abundance of TUNEL positive cells in wear debris-induced interface membranes (Fig. 2b). Results of remaining tissues are summarized in Fig. 5.

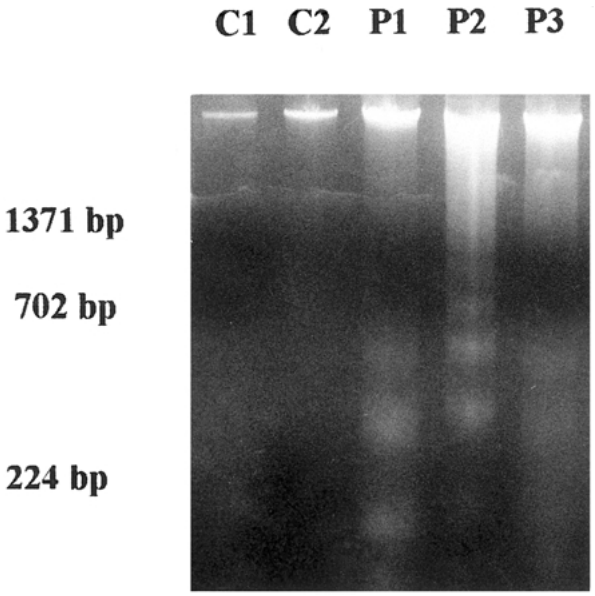


Figure 3 DNA laddering in interface membranes of failed THAs. Genomic DNA was extracted from control and interface membrane tissues and analyzed on 1.5% agarose gels. Lambda DNA digest was used as a molecular marker. Control tissue (lanes C1 and C2) show absence of laddering, while wear debris-induced interface membranes (lanes P1, P2 and P3) demonstrate positive DNA laddering, a characteristic feature of apoptosis. Results of remaining tissues are summarized in Fig. 5.

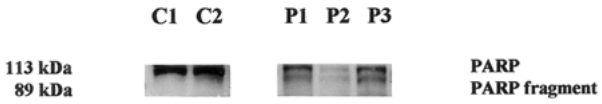


Figure 4 Expression of PARP protein in interface membranes of failed THAs. Proteins from control (C1–C2) and interface membrane (P1–P2–P3) tissues were extracted and analyzed on 10% acrylamide gels. Apoptosis is associated with proteolysis of native 113 kDa PARP into the 89 kDa fragment. Control tissues (lanes C1 and C2) lack the 89 kDa fragment whereas the wear debris-induced interface membranes (lanes P1, P2 and P3) demonstrate the presence of apoptosis-associated 89 kDa fragment. Results of remaining tissues are summarized in Fig. 5.

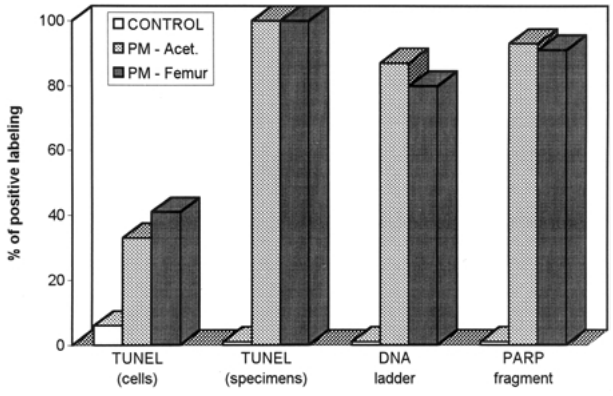


Figure 5 Apoptosis in interface membranes of failed THAs. The figure presents the summary of results for all specimens, expressed as the percentage of positive labeling for TUNEL staining, DNA laddering and expression of PARP fragment.

form and the 89 kDa proteolytic fragment of PARP. This 89 kDa proteolytic fragment was observed in 92% of specimens (30/33 specimens). It was absent in control tissues where only the native 113 kDa native form was expressed. The incidence of the 89 kDa PARP fragment was similar on both acetabular and femoral sides of the prostheses (Fig. 5).

4. Discussion

Using multiple techniques for the detection of apoptosis we have demonstrated the presence of apoptotic cells in interface membranes of failed THAs. We first demonstrated the presence of apoptosis by TUNEL stain. This increasingly applied assay is used to investigate active cell death and has the advantage of visualization at the single-cell level [26]. While TUNEL assay remains a powerful tool for the identification of apoptosis, it can at times give false positive staining late in the stages of necrosis [29]. To further confirm our identification of apoptosis, we performed DNA analysis on agarose gel, a technique considered to be the gold standard for the identification of apoptosis [30]. Our results using DNA laddering confirmed a high incidence of apoptosis.

Finally, we identified the presence of PARP fragments in PM to confirm the presence of apoptosis. PARP is a 113 kDa DNA repair enzyme whose activity is inactivated during apoptosis by some members of the caspase family [31]. Specific proteolysis of PARP into an 89 kDa fragment has been closely associated to apoptosis and its identification serves as a reliable marker of apoptosis [32, 33]. The clear identification of PARP protein in 92% of our specimens compliments our TUNEL and DNA laddering results.

The significance of identifying both modes of cell death in periprosthetic tissue, necrosis and apoptosis, is only now becoming apparent. Using a cell culture model, Catelas *et al*. [24] have shown for ceramic and polyethylene particles and Granchi *et al*. [23] have shown for metal ions that macrophage apoptosis can be induced. Catelas *et al*. [24] showed that the degree to which apoptosis occurred was directly correlated to particle size and concentration. Granchi *et al*. [23] have shown that while large amounts of nickel and cobalt produced necrosis, chromium or limited amounts of nickel and cobalt preferentially induced apoptosis, suggesting an adaptation of the tissue to the implant without the subsequent destructive inflammatory tissue reaction.

Indeed, we feel that by confirming apoptotic cell death in periprosthetic tissues we have identified an internal control mechanism that attempts to keep the destructive effects of wear debris particles in check. Our combined *in vitro* and *in vivo* findings strongly suggest that the therapeutic preferential induction of apoptotic macrophage cell death may create a more favorable periprosthetic environment in the presence of particulate debris. In this regard, bisphosphonates have been shown to promote apoptosis in murine osteoclasts [18]. It has also been recently demonstrated that bisphosphonates specifically inhibit *in vitro* the proliferation of mouse macrophages [34], probably by promoting apoptosis [35, 36]. Therefore, the presence of apoptosis in interface membranes and its possible therapeutic modulation may prove to be a key element in the prevention and/or the treatment of periprosthetic osteolysis.

In conclusion, the demonstration of apoptotic cell death in interface membranes of failed THAs in this study, combined with the potential of the bisphosphonates in the inhibition of periprosthetic osteolysis, suggest that aseptic loosening can be specifically targeted by therapeutic modulation of apoptotic pathways.

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