In situ localization of apoptotic changes in the interface membrane of aseptically loosened orthopaedic implants

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Twenty specimens of bone-implant interface membrane from THR/TKR were used for *in situ* localization of apoptotic changes. A panel of antibodies was used to label leukocyte antigens (CD68 and CD3) cytokines (IL-1α and IL-1β) and apoptosis inhibiting and promoting proteins (bcl-2 and bax) by means of immunohistochemical techniques. A DNA fragment test on the tissue sections was also carried out to confirm actual cell death using the enzyme terminal deoxy nucleotidyl transferase (TdT) to incorporate biotinylated nucleotide with the 3′-OH DNA ends. Leukocyte antigen staining showed that there were large numbers of CD68 positive macrophages as well as multinucleate giant cells (MNGC) but that CD3 positive lymphocytes were also present in the interface membrane. The leukocyte surface antigen staining pattern corresponded to previous findings [1]. Immunostaining with bcl-2 and bax antibodies revealed that both of these proteins were expressed in the cytoplasm of the cells in the interface membrane but they showed different cellular patterns. Bcl-2 was localized in a small number of lymphocyte-like cells while bax was expressed by large numbers of cells, mainly macrophages. The number of cells which expressed bcl-2 was significantly lower than that of bax ($P < 0.01$). DNA fragment localization occurred mostly in a layer of cells ($1 \sim 3$ cells deep) next to the implant surface. Again the level of DNA fragment-containing cells was significantly lower than that of bax positive cells ($P < 0.01$). The results, for the first time, indicate that there is an apoptotic activity occurring in cells in the interface membrane, but not all the cells which express apoptosis-promoting protein (i.e. bax) will enter into the phase of cell death.

1. Introduction

Aseptic loosening of orthopaedic implants used in joint replacement results from bone loss that occurs through the resorptive activity of inflammatory cells recruited in response to the presence of fine wear particles [1]. Studies of the interface membrane, the fibrous tissue between implant and bone, have shown the presence of macrophages, lymphocytes and mast cells in this inflammatory reaction. These cells produce a considerable number of chemical mediators, cytokines and growth factors, which are responsible for modulating the inflammatory process. The question arises as to the turnover and fate of these cells.

Control of cell populations in inflammation under the influence of cytokines may be by a process called apoptosis, or programmed cell death. In apoptosis, there is loss of specialized cell surface structures, reduction in cell volume with compaction of organelles and splitting of the cell into membrane-bound bodies. The chromatin of the nucleus condenses and the DNA is fragmented. The apoptotic cell, and the apoptotic bodies into which it is broken down, is recognized by macrophages and phagocytosed. This method of removing cells that are excessive to need or effete is different from necrosis which results from severely disturbed local extracellular environmental conditions such as the presence of infection, loss of blood supply or trauma. Cell death by apoptosis in contrast, is part of a regulated process [2, 3].

It is not clear whether the process of apoptosis occurs in the tissues adjacent to an orthopaedic implant. Apoptosis is controlled by intracellular proteins including bcl-2 which is anti-apoptotic and bax which promotes programmed cell death [4, 5]. In order to see whether apoptosis was occurring in the interface membrane this study set out to localize bcl-2 and bax expression by cells in the inflammatory infiltrate using immunohistochemistry. A method for detecting DNA fragments was also used to confirm actual cell death.

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2. Materials and methods

2.1. Specimens

Twenty specimens of bone–implant interface membrane from total hip/knee replacements (THR/TKR) were taken at the time of revision surgery for aseptic loosening (Table I). The specimens were snap frozen in isopentane/liquid N_2 and stored at $-70\,^{\circ}C$. Frozen sections were cut 5 μm thick before use.

2.2. Immunohistochemistry

Cryostat sections were fixed in 2% formaldehyde/10 mM phosphate-buffered saline (PBS) pH 7.4 at 4 °C for 8 min followed by a three-times wash in PBS/0.1% saponin solution. Immunostaining was then carried out to detect the presence of macrophage and T-lymphocyte surface antigens (CD68 and CD3) and expression of apoptotic related proteins (bcl-2 and bax) and cytokines (interleukin (IL)-1α and IL-1β) using a biotin/streptavidin alkaline phosphatase (AKP) technique.

Sections were incubated with the primary antibodies at 4 °C overnight (Table II). This was followed by a 1 h incubation at room temperature with biotinylated horse anti-mouse or goat antirabbit immunoglobulin (IgG) antibodies at a 1 : 100 dilution (Vector Laboratories, UK). After incubation with the secondary antibodies, the AKP streptavidin conjugate 1 : 100 was added for a 30 min incubation. The antibodies were diluted in a PBS/saponin/0.05% bovine serum albumin solution and the conjugate was diluted in a 5 mM Tris-buffered saline (TBS)/saponin pH 7.4. Each incubation was followed by three washes in PBS/saponin or TBS/saponin for 5 min each. The color reaction was developed using 5 mg napthol AS-BI phosphate (Sigma) dissolved in 200 μl dimethylformamide. This was then mixed with 10 ml 0.1 M Tris–HCl buffer pH 8.2 and 10 mg Fast Red TR salt (Sigma). Levamisole was added to the final developing solution as an inhibitor of endogeneous AKP at a concentration of 1 mM. The substrate was filtered and allowed to develop on sections for a maximum of 20 min. Finally, the sections were washed and then counterstained with Mayer's haematoxylin for 5 min followed by mounting in Aquamount (BDH Merck, UK).

2.3. DNA fragment test

In situ localization of nuclear DNA fragments was also carried out on the frozen sections using a Dead End colorimetric kit (Promega, UK) following the manufacturer's instruction. Briefly, the method involved the incorporation of biotinylated nucleotide at the 3′-OH DNA ends using the enzyme terminal deoxynucleotidyl transferase (TdT). Sections were fixed in 4% formaldehyde/PBS at room temperature for 15 min. After two washes in PBS of 5 min each, the sections were permeabilized with proteinase K/PBS solution (20 $\mu g\,ml^{-1}$) for 15 min. Equilibration buffer was applied to the sections for 10 min. Sections were then incubated with a TdT reaction mix solution at 37 °C for 1 h after removal of the equilibration buffer. The reaction was stopped by immersing the sections in 2 × saline sodium citrate (SSC) solution at room temperature for 15 min. Following two washes in TBS, the AKP streptavidin conjugate was added and the color reaction was developed as mentioned above (see Section 2.2. Immunohistochemistry).

2.4. Controls

Human tonsil was provided as positive tissue controls in both immunohistochemistry and DNA fragment tests. In the case of immunohistochemistry, negative controls were achieved by replacing the primary antiodies with the antibody diluent or non-immune IgG from the same species. Omission of TdT was used for the negative control in the DNA fragment test.

TABLE I Details of patients and their specimens used in the present study

Patient ID#	Specimen	Age	Sex	Underlying disease	Duration of implant (yrs)	Revision	Joint replaced	Implant materials
159/94	FIM	59	F	OA	7	1	THR	Ti (UC)
187/94	FIM	57	F	OA	3	1	THR	Ti
9/96	FIM	70	F	OA	10	1	THR	Co-Cr(C)
79/96	FIM	61	F	OA	7	2	THR	Ti (UC)
83/96	FIM	66	M	OA	4	2	THR	Ti (UC)
9/97	FIM	63	F	OA	12	2	THR	Co-Cr(C)
10/97	FIM	55	M	OA	15	1	THR	Co-Cr(C)
11/97	FIM	60	F	OA	14	1	THR	Co-Cr(C)
12/97	FIM	71	F	OA	15	1	THR	Co-Cr(UC)
13/97	FIM	66	F	RA	8	2	THR	Ti (C)
15/97	FIM	55	M	OA	11	1	THR	Co-Cr(C)
64/97	AIM	46	F	OA	4	1	THR	HA-coated Co-Cr
65a/97	FIM	49	F	OA	5	1	THR	Co-Cr(UC)
71/97	FIM	79	F	OA	5	1	THR	Co-Cr(C)
200/97	FIM	48	M	OA	4	2	THR	HA-coated Co-Cr
273/97	FIM	77	F	RA	10	1	TKR	HA coated Co-Cr
345/97	AIM	60	M	OA	6	2	THR	HA-coated Co-Cr
346/97	FIM	82	F	OA	3	2	TKR	Co-Cr(UC)
365/97	FIM	79	M	OA	8	1	THR	Co-Cr(UC)
367/97	FIM	64	M	OA	2	2	THR	HA-coated Co-Cr

FIM = femoral interface membrane; AIM = acetabular interface membrane; OA = osteoarthritis; RA = rheumatoid arthritis; THR = total hip replacement; TKR = total knee replacement; Ti = TiAlV; CoCr = Co Cr Mo; UC = uncemented; C = cemented; HA = hydroxyapatite.

TABLE II Primary antibodies used in this study

Antibody	Specificity	Isotype	Clone	Host species	Company	Working dilution
CD68	Human macrophage-associated CD68	IgG1	Monoclone (KP1)	Mouse	DAKO	1 : 200
CD3	Human T-cell associated CD3 antigen	IgG1	Monoclone (UCHT1)	Mouse	DAKO	1 : 25
bcl-2	Human bcl-2 oncoprotein	IgG1	Monoclone (124)	Mouse	DAKO	1 : 50
bax	Human bcl-2 family protein bax	IgG	Polyclone	Rabbit	Oncogene	1 : 50
IL-1α	Human IL-1α	IgG	Polyclone	Rabbit	Genzyme	1 : 100
IL-1β	Human IL-1β	IgG/IgM	Polyclone	Rabbit	Genzyme	1 : 100

2.5. Microscopical examination

The intensity of immunostaining with all the antibodies was evaluated by scoring the staining reaction in four groups: 0 = no cytoplasmic staining; 1 = weak cytoplasmic staining intensity; 2 = moderate cytoplasmic staining intensity; 3 = strong cytoplasmic staining intensity. The quantity of the positive staining cells was evaluated as the percentage of the total number of cells showing cytoplasmic positivity.

The same scoring system was also used to evaluate the staining intensity of nuclei in the DNA fragment test and the quantity of the positively stained cells expressed as a percentage.

2.6. Statistical analysis

Microsoft Excel for Windows was used as the spreadsheet for descriptive statistics (means and standard errors of mean). The significance of the associations was determined using the two-tailed Student's *t*-test. Probability values $P \leq 0.05$ were considered as statistical significance.

3. Results

Leukocyte surface antigen staining showed that there were large numbers of CD68 positive macrophages as well as multinucleate giant cells (MNGC) but that CD3-positive T lymphocytes were also present in the interface membrane (Fig. 1a, b). Fifteen out of 20 samples expressed IL-1α in the cytoplasm of cells in the interface membrane and 17 cases showed IL-1β positive cells. The cell population of IL-1 expression corresponded to that of CD68.

Seventy per cent of the cases expressed the bcl-2 family proteins, bcl-2 and bax in the cytoplasm of cells in the interface membrane but the staining patterns for these two proteins were very different. Bcl-2 was localized in a small proportion of lymphocytes and mostly in a scattered manner (Fig. 2a), whereas bax was expressed by a variety of cells. Of the bax positive cells, macrophages were the main cell population (Fig. 2b). Some endothelial cells showed bax staining in the cytoplasm as well. Most MNGC showed weak or no expression of bax.

The DNA fragment test showed that nuclear DNA fragments occurred in 85% of cases. Interestingly, the DNA fragment localization occurred mostly in a layer of cells (one to three cells deep) next to the implant surface. Occasionally, in the sublining layer or deeper interface membrane, some MNGCs containing metal or polyethylene debris showed DNA fragment staining. A small number of T lymphocytes were also DNA fragment positive. The staining intensity varied between individual cells (Fig. 3).

A summary of the results is shown in Table III. There was no staining in the negative controls. The positive tissue control of human tonsil showed that bcl-2 was stained in interfollicular T lymphocytes, while bax and DNA fragment staining was seen in some B lymphocytes within germinal centers.

4. Discussion

The present study demonstrated that a large number of macrophages were present in the interface membrane in almost every case, as well as some T lymphocytes. In

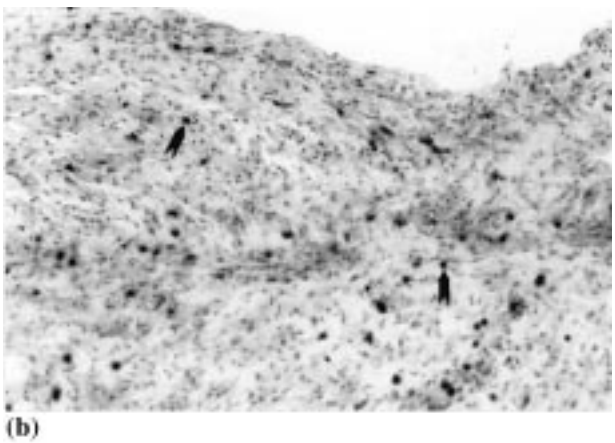
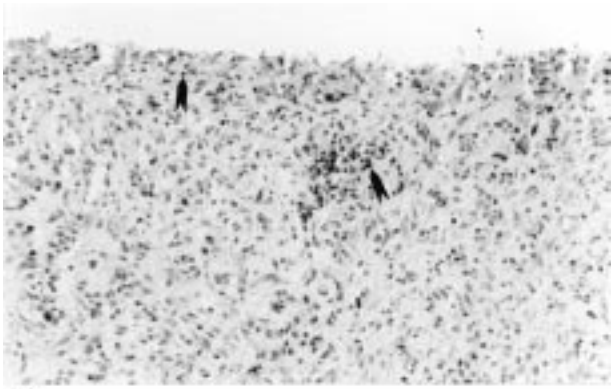


Figure 1 (a) Photomicrograph of the synovium-like layer in the interface membrane next to an implant shows macrophages (white arrowheads) and MNGCs (black arrowheads) which are positively stained with CD68 monoclonal antibody (original magnification = × 250). (b) Photomicrograph of the interface membrane, shows CD3 positive T lymphocytes in a scattered manner (arrowheads) (original magnification = × 400).



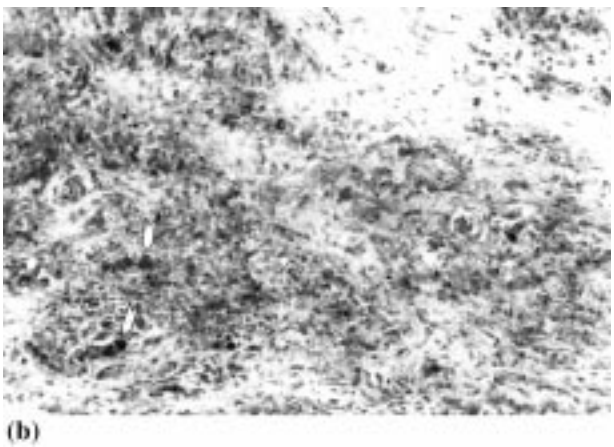


Figure 2 (a) Photomicrograph of immunostaining in the interface membrane, shows that a number of lymphocyte-like cells are positively labeled for bcl-2 (arrowheads) (original magnification = × 250). (b) Photomicrograph of immunostaining in the interface membrane shows strong reactivity of bax in the cytoplasm of macrophages (arrowheads) (original magnification = × 250).

addition, most of these cells were expressing cytokines IL-1α and IL-1β. These immunostaining results correspond to the previous findings from our group [1]. In recent years, it has been reported that a number of cytokines such as tumor necrosis factor-α (TNF-α), IL-1, IL-6 and granulocyte–macrophage colony-stimulation factor (GM-CSF) are present in the interface membrane [1]. Apart from the fact that these cytokines are inflammatory mediators, TNF-α and IL-1β are known to be involved in the process of apoptosis [6, 7]. TNF-α is one of the important extracellular activators of apoptosis in the mammalian immune system [6]. IL-1β is the product of activated ICE (IL-1β converting enzyme), a crucial enzyme in the induction of apoptotic cell death [6]. The activated ICE uses non-functional pro-IL-1β as its substrate and converts it into biologically active IL-1β [7].

It is now well known that bcl-2 family members are one of the most important components of the apoptotic regulatory machinery [8]. Bcl-2-related proteins either suppress or promote apoptosis by interacting with or functionally antagonizing each other [9, 10]. In an excess of bcl-2, bcl-2/bax heterodimers are formed, which leads to prevention of cells undergoing programmed death. Conversely, where there is dissociation of bax from bcl-2 or an excess of bax, bax homodimers predominate, which favors apoptosis. The present study showed that a large population of macrophages in the interface expressed bax protein, a smaller proportion of cells expressed bcl-2 and the percentage compared with bax expressing cells was significantly lower ($P < 0.01$; see Table III). This indicates that there is an apoptotic activity occurring in these cells. This process is regulated by gene products [10] and is distinctly different from cell death occurring by necrosis, which is also sometimes seen in interface tissues [11].

The DNA fragment test provided further evidence of the programmed cells death [3] present in the interface membrane. However, the statistically significant differences ($P < 0.01$; see Table III) in positive cell numbers between bax and DNA fragment containing cells indicate that not all bax positive cells have entered the final phase of cell death. Cells showing bax expression and DNA fragmentation were predominantly situated in the surface layer near to the implant. Deeper bax-positive cells may

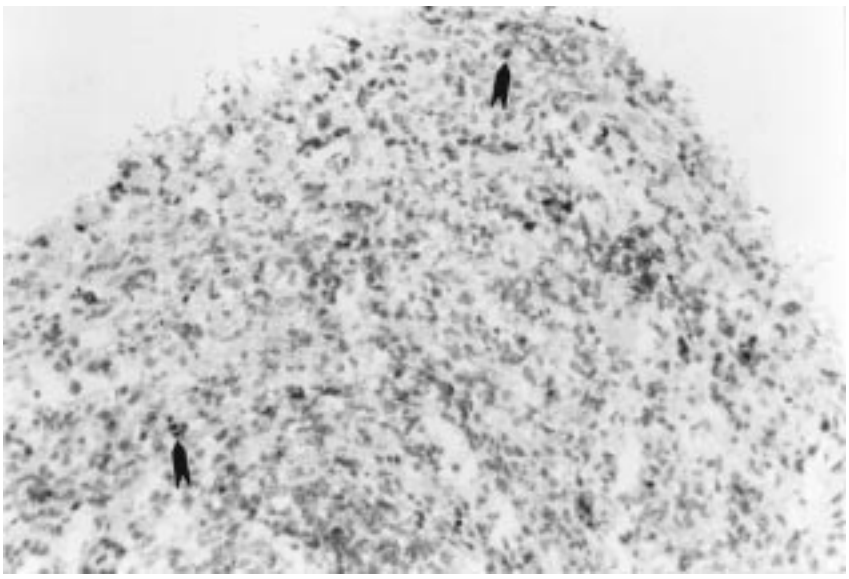


Figure 3 Photomicrograph of the interface membrane next to an implant shows a small number of cells containing DNA fragments (arrowheads) (original magnification = × 250).

TABLE III Immunocytochemical expression of CD68, CD3, IL-1, bcl2, bax and demonstration of DNA fragment in interface membrane of aseptically loosened prosthesis

Examination	CD68	CD3	IL-1α	IL-1β	bcl-2	bax	DNA fragment
No. of cases (%)	20(100)	18(90)	15(75)	17(85)	14(70)	14(70)	17(85)
Mean % of cells (± SEM)	39.25 ± 6.55	14 ± 1.97	32 ± 7.66	36 ± 7.55	6.3 ± 1.52	23 ± 5.74	5.25 ± 0.88
Mean score (± SEM)	2.6 ± 0.17	2.7 ± 0.21	1.55 ± 0.27	1.7 ± 0.25	1.7 ± 0.28	1.95 ± 0.31	1.8 ± 0.21

be reverse regulated by bcl-2 expression mostly from T lymphocytes.

5. Conclusion

This paper has, for the first time, reported that apoptosis occurs in the interface membrane of aseptic loosening from THR/TKR based on the immunohistochemical and DNA fragment findings.

Further investigation is needed in elucidation of the mechanism of apoptotic change caused by implant wear particles in the interface membrane.

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