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Fibroblast apoptosis and caspase-8 activation in aseptic loosening

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Abstract

The presence of apoptosis has been investigated in the interface membranes collected during revision surgery of loosened total hip joint arthroplasty (THAs). Terminal deoxyrobonucleotidyl transferase (TdT) assay for apoptotic DNA fragmentation quantification revealed a statistically significant presence of apoptosis in aseptic samples, obtained from both cementless ($2.37 \pm 0.6\%$) and cemented ($12.01 \pm 1\%$) prosthesis compared to septic samples where apoptosis was almost absent. Activated caspase-8 immunostaining was almost undetectable in septic samples, while in the aseptic samples active caspase-8 was present weakly in the cementless samples ($1.35 \pm 0.22\%$) and strongly in the cemented ones ($9.0 \pm 0.40\%$). The caspase-8 cytoplasmatic staining allowed the morphological recognition of positive cells both as fibroblast-like and immunocompetent cells. In aseptic cemented samples fibroblast-like cells were the most represented subpopulation in the caspase-8 positive population scored (76.6%) compared to the immunocompetent cells (23.4%).

Caspase-8 activation is an upstream event in the apoptotic pathway triggered by the activation of cytokines receptors such as TNF-α receptor 1 (TNFR-1), and the presence of caspase-8 activation in fibroblast-like cells in the aseptic interface membranes of THAs suggests a possible TNF-α dependent apoptosis.
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Keywords: Total hip prosthesis; Aseptic loosening; Apoptosis; Caspase-8

1. Introduction

Aseptic loosening of orthopedic implants used in joint replacement results from bone loss that occurs through the resorptive activity of inflammatory cells activated by the presence of wear particles [1]. Inflammatory cells, mainly macrophages, lymphocytes and mast cells have been observed in this inflammatory reaction at the interface membrane and it has been demonstrated that in vitro polyethyene, ceramics and metal wear particles induce macrophage apoptosis [2,3]. Apoptosis has been observed in the periprosthetic site and it has been interpreted as a sign of resolution of inflammation [4]. Apoptosis is a physiological, gene-directed form of mechanism of programmed cell death aimed to the maintenance of tissue homeostasis [5]. Apoptosis plays a crucial role in modulating tissue growth during embryonic development and cell turnover in adult life, particularly in immunological and hormone-dependent systems [6]. Cells appear to initiate their own apoptotic death through the activation of endogenous proteases called caspases. Their activation results in cytoskeletal disruption, cell shrinkage, and membrane blebbing. Apoptosis also involves characteristic changes within the nucleus that undergoes condensation and DNA is degraded into DNA fragments the size of oligonucleosomes [7]. The presence of apoptotic cell death has been detected in interface membrane the fibrous tissue between implant and bone, of aseptically loose total hip arthroplasties (THAs) suggesting that apoptosis-related events are indeed associated with periprosthetic osteolysis [8].

In order to investigate the presence and the mechanisms of apoptosis at the periprosthetic sites, apoptotic cells have been quantified using both terminal deoxyrobonucleotidyl transferase (TdT) assay for DNA fragmentation quantification and active caspase-8 immunostaining in implant tissues collected during revision surgery of loosened hip joint prostheses.

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2. Materials and methods

2.1. Specimen collection

Twelve specimens of bone implant interface membrane from cementless and cemented total hip replacement (THAs) were taken at the time of revision surgery for septic (cemented, $n = 2$; cementless = 2) and aseptic (cemented, $n = 4$; cementless, $n = 4$) loosening at the CTO, Dept. of Orthopedics, Traumatology and Occupational Medicine, University of Turin, Italy.

2.2. Tissue processing

Each specimen was washed in cold phosphate-buffered saline (PBS) and fixed in 10% buffered formalin. Specimens were then dehydrated through ascending series of ethanol, cleared in xylen and embedded in semi-synthetic paraffin (Sherwood Medical Co. St. Louis, MO, USA).

Entire paraffin blocks were cut serially with a rotatory microtome (Leica-Jung, Germany) in consecutive 5 μm thick sections, and subdivided for morphological analysis in groups of ten section each.

2.3. TUNEL technique

For in situ TUNEL technique, an Apoptag® kit (Intergen Company, New York, USA) was used. Five sections for sample(s) were incubated with proteinase-K for 15 min at room temperature, washed with phosphate-buffered saline (PBS) (pH 7.2–7.4) and quenched in 1% H_2O_2 in PBS for 5 min followed by three washes in PBS. They were then incubated with equilibrating buffer for 15 s. After draining off excess buffer, sections were treated with 54 μl of terminal deoxyrobonucleotidyl transferase (TdT) enzyme for 1 h at 37°C, labelled nick-end tail with digoxigenin-dNTP, incubated for 10 min at room temperature in the stop-wash buffer and incubated for 30 min at room temperature with anti-digoxigenin peroxidase conjugate antibody. At the end of incubation sections were washed again with PBS and exposed to diaminobenzidine (DAB) solution to developed reaction product. After washing in distilled water, sections were counter-stained with Methyl Green, dehydrated and mounted in a semi-synthetic mounting medium.

The percentage of apoptotic cells per section was determined by the ratio of TUNEL positive nuclei/total nuclei number. Ten microscopic fields of 50–200 nuclei (400 × magnification) on five different sections were analyzed for each sample.

2.4. Caspase-8 immunohistochemistry

Five sections for each sample were processed for caspase-8 immunohistochemistry by exposing them to rabbit monoclonal antibody raised against activated caspase-8 (Upstate biotech, NY, USA) at a concentration of 1 μg/ml. The section were hydrated, and incubated in a specific serum for 1 h at room temperature. Primary antibody incubation was done overnight in a humid chamber at 4°C. The excess of unbound antibody was removed by washing sections twice in the buffer solution. The product of immunoreaction was revealed by incubating sections with anti-rabbit secondary antibody complexed with avidin-biotin system and evidenced by DAB reaction (ABC Staining System kit, Santa Cruz, CA, USA; sc-2018). Control sections were processed as above, but using a non-immune mouse IgG instead of primary antibody, or by omitting the primary antibody from incubation medium. In these conditions no specific immunostaining was observed.

The percentage of caspase positive cells per section was determined by the ratio of caspase positive cell/total cell number. Caspase positive cells were also identified on the basis of morphology as fibroblast-like cells (cell presenting elongated body cell profile resident between collagen fibers bundles) or immunocompetent cells (cell with a small round body). Ten microscopic fields of 50–200 nuclei (400 × magnification) on five different sections were analyzed for each sample.

2.5. Data analysis

Means of different parameters investigated were calculated from single sample data, and group means ± S.E.M, were then obtained from single sample values. Statistical analysis of variance (ANOVA) was used; the significance of differences between means was assessed by Newman–Keuls multiple range test, taking $p<0.05$ as the minimum level of significance.

3. Results

3.1. TdT analysis of apoptosis

TUNEL reaction was localized in cell nuclei present in the perimplantal tissue obtained from failed implants, both aseptic and septic (Fig. 1). In the septic samples very few apoptotic cells were counted and the percentage of TdT positive cells observed was 1.12 ± 0.3% and 0.8 ± 0.4% for cementless (SNC) and cemented (SC) samples respectively. In the aseptic samples the number of apoptotic cells raised to a percentage of 2.37 ± 0.6% ($p<0.05$) and 12.01 ± 1% ($p<0.001$) respectively in cementless (ANC) and cemented (AC) samples. This increase in the number of TdT-positive cells in the aseptic samples (Fig. 2) was particularly evident in the samples from cemented prosthesis (Fig. 2B) compared to the ones from cementless samples (Fig. 2A).

3.2. Caspase-8 activation in fibroblast

The caspase-8 positivity profile observed for the four categories of samples was similar to the one obtained for TdT positivity (Fig. 3).

In fact, the positivity observed in the septic samples was almost undetectable (0.7±0.21% and 0.5±0.23% for SNC and SC samples respectively) (Fig. 3), while in the aseptic samples caspase-8 was active weakly in the cementless samples (ANC) (1.35±0.22, $p<0.05$) and strongly in the cemented ones (AC) (9.0±0.40%, $p<0.001$). As shown in Fig. 4A and B, the caspase-8 staining in the ANC (A) and AC (B) samples was dispersed in the cell cytoplasm allowing a clear recognition of the cell shape. Caspase-8 positive cells have been divided in fibroblast-like (elongated cell body) and immunocompetent (round cell body) cells (Fig. 4C and D) on the basis of cell shape. As shown in Fig. 5 the majority of the caspase–8 positive cells scored both in ANC and AC were fibroblast-like cells as observed on the basis of their morphology (76.6% of caspase-8 positive cells). Moreover caspase-8 positive fibroblast-like cells accounted for the 16.4±2.1% of the total fibroblast-like population (data not shown).

4. Discussion

Loosening of total hip prosthesis is a critical phenomenon in the treatment of total hip arthroplasty and it can be induced by a chronic infection or aseptically by both mechanical stress and local host response to wear debris of high density polyethylene, metals and bone cement [9]. Studies of the retrieved periprosthetic membrane, the fibrous tissue formed at the bone–implant interface, have identified the presence of apoptotic cells in this neoformed tissue. These studies have been performed mainly in membranes derived from aseptic loosening and apoptotic cells have not been clearly phenotypized [4,8,10]. Moreover the apoptosis triggering mechanism and the consequences of the phenomenon in the periprosthetic tissue have not been fully elucidated.

In our study, we observed that apoptosis, identified on the basis of late phase of DNA fragmentation, is almost undetectable in samples from septic loosening, while appears significant in samples from aseptic loosening in accordance with previous findings that observed a necrotic instead of apoptotic evolution of periprosthetic tissue in the presence of chronic infection [9].

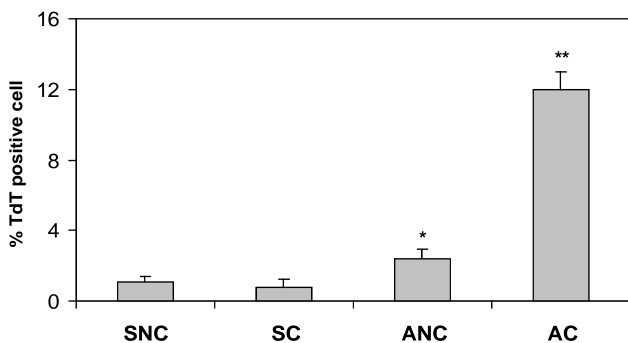

Fig. 1. Apoptosis in interface membrane of failed THAs. Results for all specimens are expressed as the percentage of positive labelling for TUNEL staining. SNC = septic cementless, SC = septic cemented, ANC = aseptic cementless, AC = aseptic cemented. *$p<0.05$, **$p<0.001$.

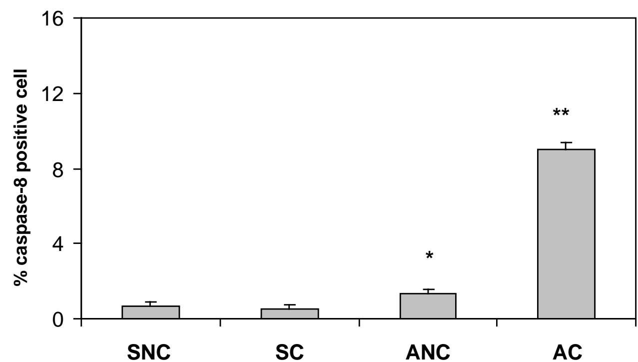

Fig. 3. Caspase-8 activation in interface of failed THA. The figure presents the summary of results for all specimens, expressed as the percentage of positive labelling for anti-activated Caspase-8 staining. SNC = septic cementless, SC = septic cemented, ANC = aseptic cementless, AC = aseptic cemented. *$p<0.05$, **$p<0.001$.

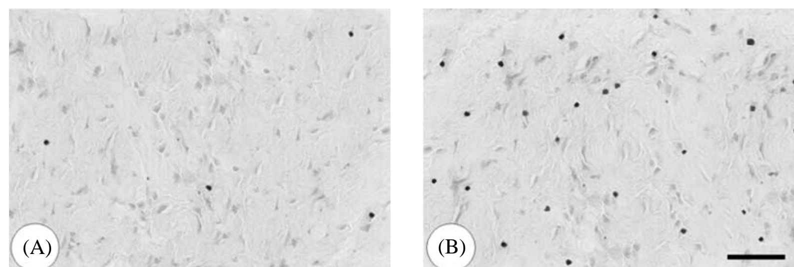

Fig. 2. In situ labelling of apoptosis-induced strand breaks in interface membranes of aseptically failed THAs. Relatively few positively stained cells were observed in cementless THA samples (A) compared to the cemented ones (B). Bar scale = 65 μm.

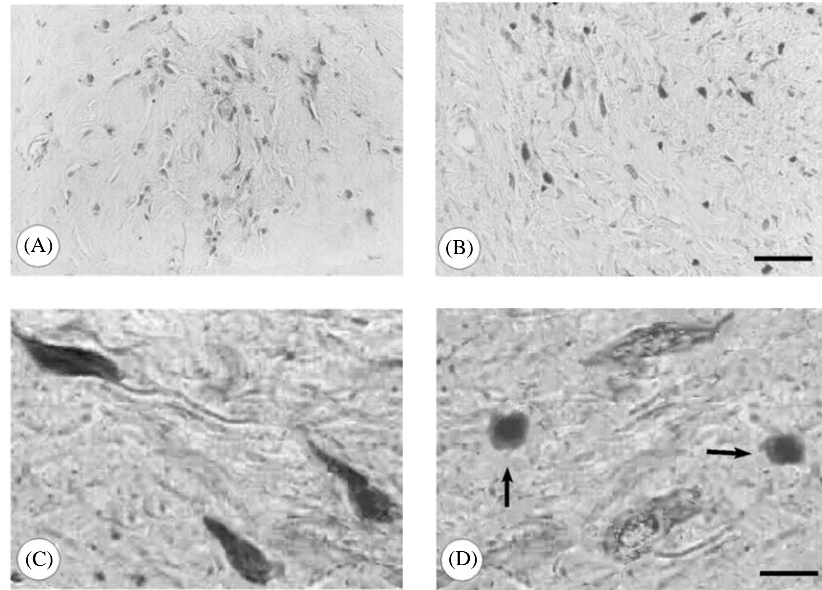

Fig. 4. In situ labelling for caspase-8 activation in interface membranes of aseptically failed THAs. Relatively few positively stained cells were observed in cementless THA samples (A) compared to the cemented ones (B). In samples treated for anti-activated caspase-8 staining (cytoplasmatic staining) it was possible to observe both fibroblast-like cells with an elongated body (C) and immunocompetent cells with a small round body (arrows) (D). Bar scale = (A,B) 45 μm; (C,D)15 μm.

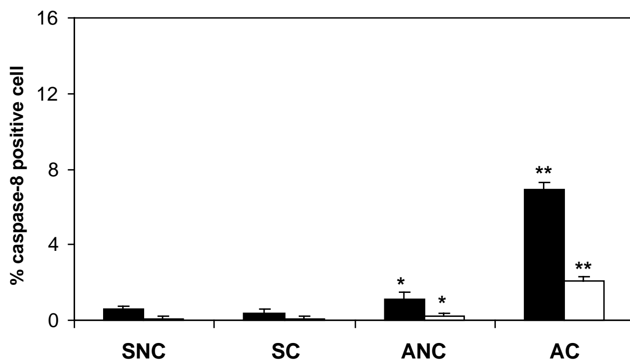

Fig. 5. Caspase-8 activation in interface membrane of failed THA. The figure presents the summary of results for all specimens, expressed as the percentage of fibroblast-like (black bar) and immunocompetent (white bar) caspase-8 positive cells scored on the basis of their morphology. SNC = septic cementless, SC = septic cemented, ANC = aseptic cementless, AC = aseptic cemented. *$p<0.05$, ** $p<0.001$.

Apoptosis in periprosthetic tissues seems to occur in cemented and cementless prosthesis without remarkable differences [4]. On the contrary in our samples the presence of cement increased the percentage of TdT positive cells. In fact bone cements have been shown to induce in vitro apoptosis in osteoblast-like and HL-60 cells [11,12]. These observations suggest that the apoptosis observed in the cementless samples could be mainly a ‘‘physiological’’ apoptosis occurring during the normal tissue turnover [5].

In the apoptotic pathways, caspase-8 acts as initiator of caspases proteolytic cascade leading to cell death [13]. Caspase-8 plays a key role in apoptosis triggered by death receptor such as Fas, TNFR-1 and DR3 [14]. Moreover caspase-8 is involved in tumor cell death induced by chemotherapeutic drugs [15]. In the periprosthetic membranes high levels of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α [16,17] are produced mainly by monocytes/macrophages [18] after contact with debris. Cytokines play a key role in the tissue response to the implant [19] and induce pro-apoptotic signals through cell receptor activation [18].

Caspase-8 immunohistochemistry was performed using a monoclonal antibody raised against the active form of enzyme in order to confirm the presence of apoptosis and to investigate the possible triggering mechanism of the phenomenon.

The caspase-8 positivity observed resembled the TdT positivity previously scored and also in this case AC samples presented a higher percentage of positive cells compared to all the other samples. Moreover, we distinguished, on a morphological basis, caspase-8 positive fibroblast-like and immunocompetent cells. The observed fibroblast apoptosis has not been described previously. In fact apoptosis in the perimplantal tissue has been described mainly as an inflammation resolutive mechanism [4]. Induction of apoptosis in fibroblast could be due to both cell activation and mechanical loading. In fact, it has been demonstrated that contraction of collagen gel induces fibroblast apoptosis through an integrin β1-dependent mechanism [20] and cyclic mechanical pressure induced cytokines production in human monocytes/macrophages in a synergistic action with debris [21]. Among cytokines, TNF-α secreted by active macrophages [22] acts modulating the fibroblast proliferation [23], inducing

apoptosis [24] and it is involved in the pathogenesis of fibrotic diseases [25]. Furthermore TNF-α is directly involved in periprosthetic osteolysis [26] and an anti-TNF-α therapy has been proposed as a clinical intervention for periprosthetic osteolysis [27]. Moreover TNF-α activates the caspase-8 pathway through the activation of TNFR-1 [28]. On the basis of our results, we hypothesize that TNF-α is responsible for the apoptosis observed in the fibroblast-like population in the periprosthetic membrane, although TNF-α levels modulation by bone cement is controversial [17,29].

In this scenario the fibroblast apoptosis could be considered as a side effect of inflammation leading to the end of the fibrotic reaction. The observed quantitative difference in the percentage of TdT-positive cells and caspase-8 positive cells could be accounted for the different temporal occurrence of the two phenomena during apoptosis [30]. In fact, caspase-8 activation is an early upstream event in the apoptosis and caspase-8 positive cells could be negative for TdT with a DNA still intact. On the other hand we could not exclude completely that caspase-8 activation is the only trigger of apoptosis in the periprosthetic tissues examined.

5. Conclusions

In conclusion, the fibroblast caspase-8 activation observed in the periprosthetic tissue from cemented prosthesis is a new element in the complex series of events occurring during aseptic loosening. The caspase-8 mediated apoptosis could be induced by high levels of TNF-α in the cemented prosthesis–tissue interface suggesting another effect of this cytokine in the aseptic loosening.

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