Chapter 7

Wear particles do not elicit an aseptic inflammatory response in fibrous tissue interfaces of loosening total hip replacements

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SUBMITTED

Abstract

Long-term survival of total hip replacements (THRs) is still impaired by aseptic loosening. Although in vitro studies have suggested that wear particles are the main cause for osteolysis and loosening, in vivo studies have not always shown this particle-driven osteolysis. Elevated levels of matrix-metalloproteinases (MMP) -2 and -9 have been found around loosened THRs, suggesting that proteolysis plays a role in osteolysis. Activity of proteases is highly regulated at the posttranslational level. Therefore, we investigated tissues around loosened THRs for activity of these enzymes in situ, using in situ zymography in combination with immunohistochemistry. MMP-9 activity was restricted to macrophages and MMP-2 activity to endothelial cells. In contrast to the literature, we did not encounter particles in association with inflammatory response or accumulations of leukocytes. Our data indicate that aseptic loosening is not particle induced, but is rather caused by processes such as increased fluid pressure causing damage to bone and interface as has been shown in animal models.

Introduction

Hip replacement surgery is a successful and cost effective procedure. Although life expectancy of hip replacements has improved over the years, aseptic loosening still poses a clinical challenge, as it remains the most common reason for revision surgery.

Processes leading to aseptic loosening, the formation of an interface membrane, osteolysis, and eventually implant loosening have not been completely unraveled yet. Osteolysis has been suggested to be the result of wear of the implant, especially of the acetabular polyethylene component. Several reports have described the inverse relationship between wear rate and survival of the implant[1-4].

Polyethylene particles have been shown to cause an inflammatory reaction leading to the activation of macrophages in vitro[5] and[6,7]. In vivo studies have shown osteoclastic bone resorption[2,8], induced by activated macrophages. However, studies in animal models have only shown decreased bone formation, suggesting that osteolysis cannot merely be induced by an inflammatory reaction to wear particles[9,10].

Total hip athroplasties cause complex reactive responses in tissues and cells[11-18]. Proteolytic enzymes are considered to play a role in the process of loosening of total hip replacements. Takagi et al[19] have shown that in periprosthetic granulomatous interface tissues, proteolytic enzymes such as MMP-1, -3 and -9 and membrane type 1 (MT-1) MMP play a role in osteolysis. It was also shown that MMP-2 and MMP-13 were present in pseudocapsular fluid in excess of their inhibitors (tissue inhibitors of MMP; TIMPs)[20].

Other proteolytic enzymes have also been demonstrated in interface membranes, such as the cysteine proteinase, cathepsin B[21], and serine proteinase cathepsine G[22]. Because proteolytic activity is highly regulated at the posttranslational level[23,24], the mere presence of proteolytic enzymes or activity in tissue extracts is of limited value in understanding the role of proteolytic enzymes in loosening of THA in particular. In situ localization of activity of MMPs, especially those associated with the breakdown of the interstitial matrix and osteolysis, can give a better insight in their role in the process of loosening of artificial joints. Determination of the activity of key proteinases in this process could lead to the development of local targeted therapy using specific proteinase inhibitors. As our understanding of the microenvironment of hip replacements improves, modification of these processes comes into view [25].

Goal of the study

To show the activity of MMP-2 and MMP-9 in situ in the interface of loosened THAs using in situ zymography in combination with the immunohistochemical localization of these gelatinases in relation with the presence of wear particles and macrophages, which are so often associated with osteolysis and loosening.

Case	gender	age (years)	type of fixation	revised compo-nents	time to revision (years)
1	F	86	Cemented	stem	11
2	F	90	Cemented	both	20
3	F	77	Cemented	both	16
4	F	76	Cemented	both	6
5	F	73	Cemented	stem	4
6	M	63	Cemented	both	15
7	M	54	Cementless	both	11
8	M	56	Cementless	stem	2
	Avg	71.875		Avg	10.625

Table 1. *List of total hip revision patients*

Material and Methods

Sampling

Synovium-like tissues were collected during revision surgery of 8 failed total hip replacements of 3 male and 5 female patients. Septic loosening was ruled out by several means, using the erythrocyte sedimentation rate, determination of the C-reactive protein (CRP) levels, macroscopic examination, tissue culturing and joint aspirates. The mean age of the patients was 71.9 years and the mean time between implantation and revision was 10.3 years. All patients were primarily operated for osteoarthritis of the hip (Table 1.). Tissue samples were frozen in liquid nitrogen and then taken to a storage freezer at -80 °C.

Histochemical analysis

Cryostat sections (thickness, 8 µm) were cut at -25°C for histochemical, immunohistochemical and in situ zymography staining. Sections were dried for 30 min at room temp and fixed either for 30 min at room temperature in 4% paraformaldehyde (Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS) and rinsed in distilled water, or for 12 min in acetone at 4°C and dried for 10 min at room temp. For orientation in the fibrous tissue interfaces, sections were stained with a Giemsa solution (Merck) and subsequently rinsed in distilled water, ethanol, and xylene. Then, sections were mounted in Euparal (Chroma, Stuttgart, Germany). Serial sections were used for immunohistochemistry and in situ zymography.

Immunohistochemical staining was performed to analyze the cellular composition of the fibrous tissue interfaces. Fibroblasts were localized with the ASO2 primary antibody (dilution, 1:400; Dianova, Hamburg, Germany), monocytes with the anti-CD64 primary antibody (dilution, 1:400; Sanquin, Amsterdam, The Netherlands), macrophages with the anti-CD68 primary antibody (dilution, 1:100; Dako, Glostrup, Denmark), B cells with the anti-CD20 antibody (dilution, 1:10; Sanquin, Amsterdam, The Netherlands), and T cells with the anti-CD3 antibody (dilution, 1:100;BD, Mountain view, CA, USA). For negative controls, sections were incubated with mouse IgG (dilution, 1:1000; Dako) instead of the primary monoclonal antibody. Air-dried sections were rinsed three times in PBS containing 1% (w/v) fetal calf serum (FCS; Hyclone, Logan, UT, USA). Then, sections were incubated with primary antibody dissolved in PBS for 2 h at room temp and rinsed again three times in PBS containing 1% FCS and incubated with rabbit anti-mouse antibodies conjugated with horseradish peroxidase (dilution, 1:50; Dako) for 60 min at room temp. Sections were rinsed again three times in PBS containing 1% FCS and peroxidase activity was visualized by incubation for 10 min at room temp in a solution containing 1 mM 3-amino-9-ethylcarbazole (AEC; Sigma, St. Louis, MO, USA), 5% (v/v) dimethylformamide, 0.05% (v/v) hydrogen peroxide (Merck) and 50 mM acetate buffer (pH 4.9). AEC was dissolved first in dimethylformamide. Hydrogen peroxide was added to the solution immediately before incubation. After incubation, sections were rinsed in distilled water, and nuclei were counterstained with a haematoxylin solution for 3 sec. After rinsing in tap water and finally distilled water, sections were mounted in glycerin-gelatin.

MMP-2 and -9 were localized immunohistochemically[26]. Primary monoclonal antibodies were anti-human MMP-2 and MMP-9 (each in dilution 1:400;

Neomarkers; Fremont, CA, USA). The immunohistochemical procedure was the same as described above for the detection of specific cell types.

Gelatinase activity was localized by in situ zymography as described by Mook et al.[27] and Frederiks and Mook[28]. Unfixed cryostat sections were dried for 30 min at room temperature. The substrate dye quenched (DQ)-gelatin (Molecular Probes, Leiden, The Netherlands) was dissolved in agarose solution and poured onto sections. After 60 min incubation at room temp, fluorescein isothiocyanide (FITC) fluorescence was present at sites of gelatinase activity. Control incubations were performed by adding 20 mM EDTA to the DQ-gelatin solution, which inhibits activity of MMPs[27].

Photomicrographs were made using standard light and fluorescence microscopy. Correlative light and electron microscopy was performed according to Vogels et al.[29,30]

The tissue block that was used for cryostat sectioning, was brought directly from storage at -80°C into fixative (1% [vol/vol] glutaraldehyde [Merck] and 4% [wt/vol] paraformaldehyde) in 0.1 M phosphate buffer (pH 7.4) at 4°C. Fixation was performed for 48 to 72 hours at 4°C under continuous rotation of the vials. After rinsing overnight in the same buffer at room temperature, the tissue blocks were postfixed in 1% (vol/vol) osmium tetroxide (Merck) in 100 mM phosphate buffer (pH 7.4) for 1 hour in the dark at room temp. Dehydration and embedding in epoxy resin LX112 (Ladd Research Industries, Burlington, VT, USA) were performed according to routine procedures.
Semithin sections were obtained from the surface of the tissue block in which parallel cryostat sections had revealed structures that warranted further EM observations. These sections were stained with toluidine blue to select an area for ultrathin sectioning. Ultrathin sections were stained with uranyl acetate (Leica, Wetzlar, Germany) and lead citrate (Leica).

Results

The fibrous tissue interfaces of all patients investigated showed similar histology. All interface tissues contained cell-rich areas, areas mainly consisting of well-vascularized and poorly vascularized areas and areas containing accumulations of wear particles that never contained cells or extracellular matrix (Fig. 1).

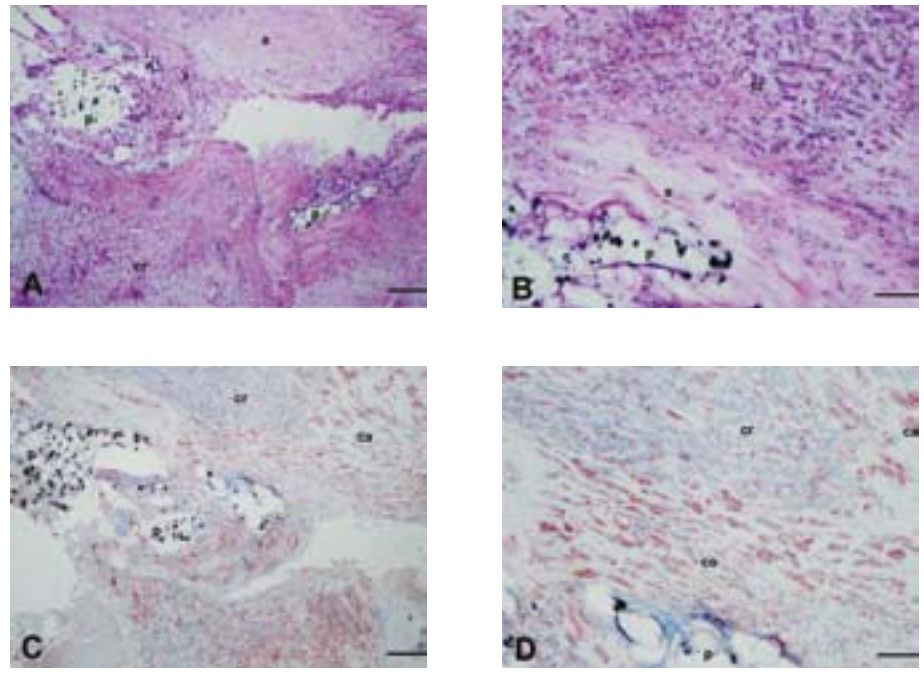

Fig. 1. *Photomicrographs of a fibrous tissue interface between bone and aseptically-loosened hip arthroplasty. Low (A) and higher power (B) overview of a Giemsa-stained cryostat section show cell-rich areas (cr), areas containing mainly extracellular matrix (e), well-vascularized areas containing capillaries (ca), larger vessels, and areas with accumulations of wear particles (p). Areas with accumulations of wear particles do not contain cells or extra-cellular matrix. Bar = 200 µm (A) and 60 µm (B), respectively. Low (C) and higher power (D, E) overviews of a cryostat section immunohistochemically-stained for MMP-2 protein. MMP-2 is present in endothelial cells of cappilaries (cap) and is associated with thick collagen bundles. Areas of wear particles do not contain MMP-2 protein. Bar = 200 µm (C)* and 60 µm (D, E), respectively. Higher power overview (F) of the localisation of MMP-9 protein. MMP-9 protein is present in a few cells only (arrows) that are not associated with accumulation of wear particles. Bar = 60 µm.

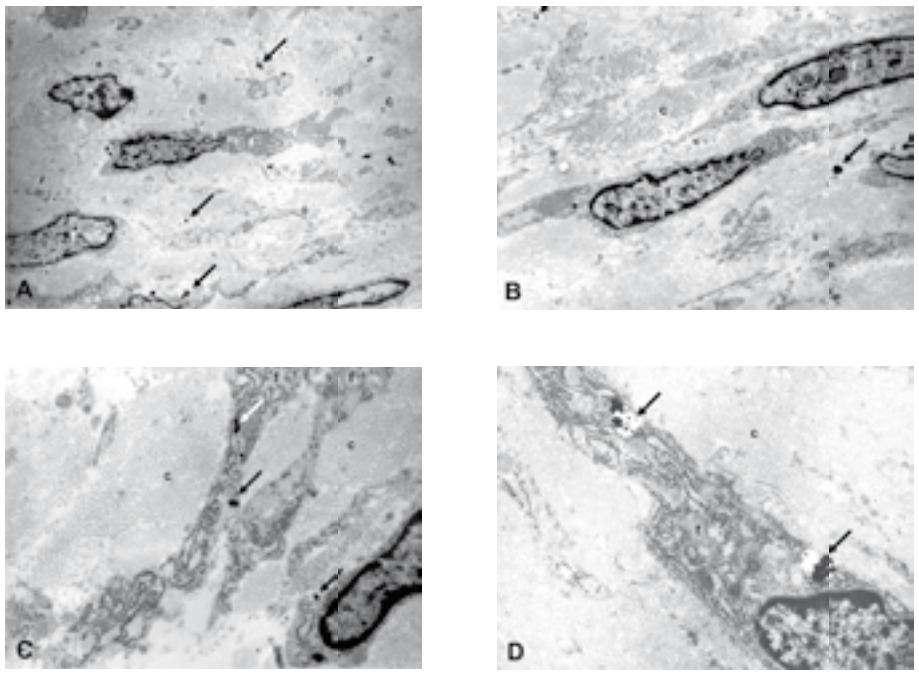

Fig. 2. *Electron micrograph of fibroblasts (f) surrounded by collagen bundles (c) in a fibrous tissue interface showing intracellular and extracellular wear particles (arrows).*

Bar= 2µm (A), 1 µm (B), 0.5µm (CD)

Its variation was not associated with either bone-facing side or arthroplasty-facing side. We did not find any area that showed signs of (aseptic) inflammation.
All tissues investigated were quiescent as is shown in Fig. 2. Fibroblasts contained internalized wear particles, whereas loose extracellular particles were present as well. These particles never induced inflammatory responses. Cells consisted mainly of fibroblasts (Fig. 3A) and relatively few macrophages (Fig. 3C), hardly any monocytes (Fig. 3B) and almost no B and T cells (data not shown). Many areas of the interface tissues were well-vascularized (Fig. 1). Endothelial cells of these vessels were strongly positive for MMP-2 protein but not MMP-9 protein (Fig. 1 C-F). This endothelium-associated MMP-2 was active (Fig. 4). MMP-2 protein was also found in the acellular areas associated with collagen bundles. This MMP-2 was active as well (Fig. 1C-E and Fig. 4). MMP-9 was present in scattered cells (Fig. 1F) and was active (Fig. 3D). Based on comparisons of immunostained sections and in situ zymograms, we conclude that these MMP9 activity-containing cells were macrophages.
However, we did not find specific areas with large amounts of inflammatory cells associated with gelatinolytic activity. Moreover, accumulations of wear particles were never associated with cells or gelatinolytic activity.

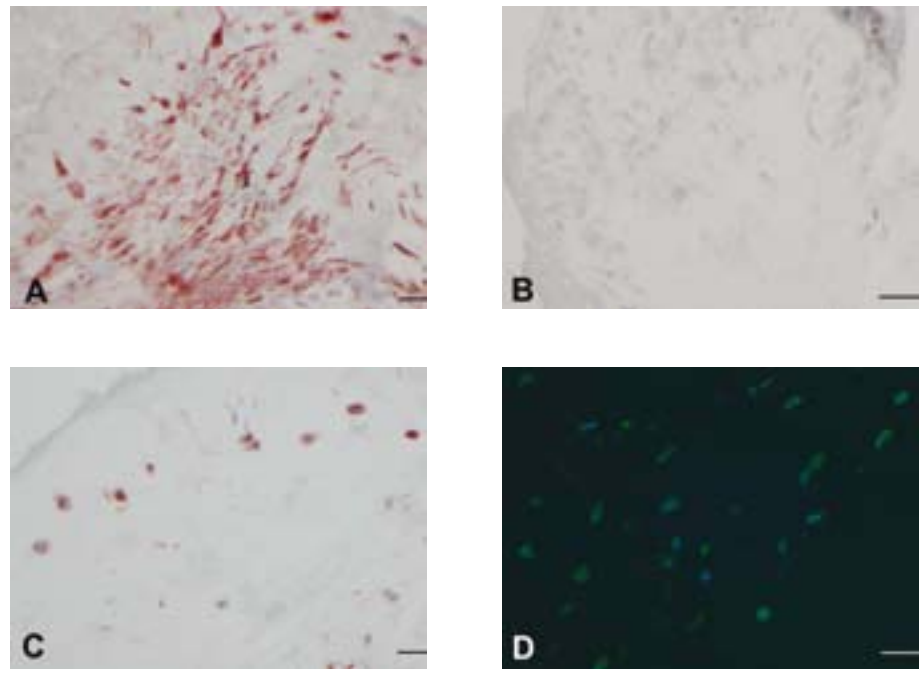

Fig. 3. *Immunohistochemical staining of fibroblasts (**A**), monocytes (**B**) and macrophages (**C**) and in situ zymography of gelatinolytic activity (**D**) in serial sections of a fibrous tissue interface. Fibroblasts are abundantly present, whereas monocytes are absent and macrophages are present in modest amounts. Gelatinolytic activity is mostly present in macrophages and not in the extracellular matrix. Bar = 60 µm (**A-D**).*

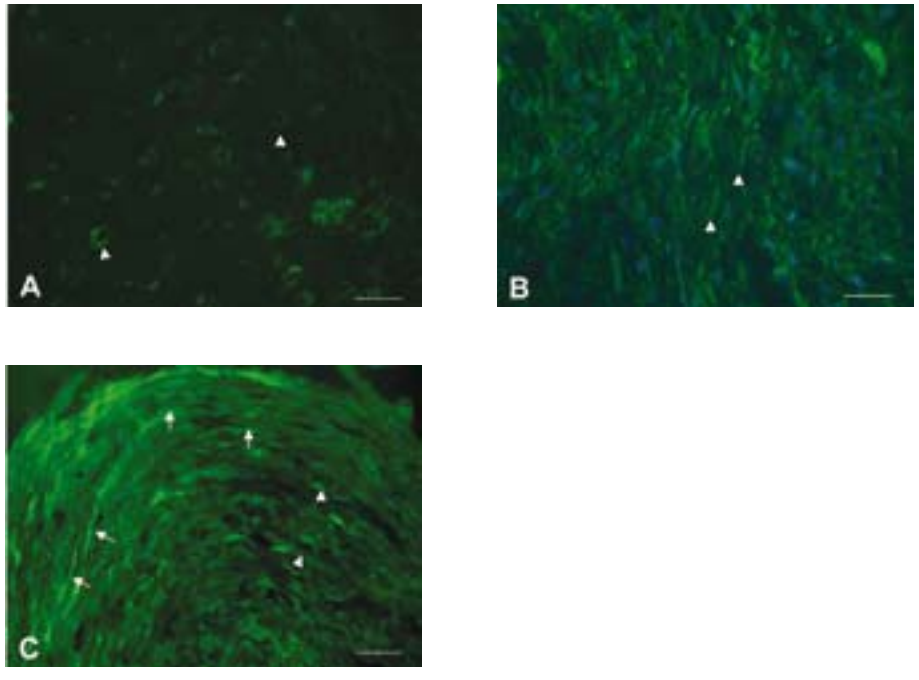

Fig 4. *Gelatinolytic activity (of MMP-2 and MMP-9) (green fluorescence) in cryostat sections of a fibreus tissue interface. Nuclei of cells are stained in blue.* **A**. *Area with activity mainly associated with endothelial cells of vessels (arrow heads).* **B**. *Area with activity mainly associated with collagen bundles (arrow heads).* **C**. *Area with high activity associated with collagen bundles (arrows) and endothelial cells in vessel walls (arrow heads). Bar = 60 µm (***A-C***).*

Discussion

A number of studies suggest an active role of proteases such as MMPs in degredation of periprosthetic bone[20,26,31-34]. Takagi et al[31] detected gelatinolytic activity of MMP-2 and MMP-9 in extracts of interface tissue around total hip replacements that were revised for loosening. No differences were found between cemented and cementless implants. In the present study, we have confirmed the presence of MMP-2 and MMP-9 in interface tissues derived from loosened THAs and have also confirmed gelatinolytic activity using zymography in situ.

We have found active MMP-9 in the interface membrane surrounding hip implants, where it is considered to degrade the extra-cellular matrix in the osteoid, facilitating resorption by osteoclasts. However, active MMP-9 was found only in dispersed macrophage-like cells, which are known to be able to differentiate into osteoclastic bone resorbing cells[35-37], but the amount of cells containing MMP-9 was modest.

MMP-2 was found in endothelial cells lining capillaries and in acellular areas associated with collagen bundles. The exact roles of MMP-2 and MMP-9 in bone remodeling around prosthetic implants remain unclear. MMP-2 is associated with tissue degradation, and a recent report indicates its involvement in bone formation as well, because deficiency of MMP-2 was found to cause defective skeletal and facial development, decreased bone mineralization, joint erosion and defects in osteoblast and osteoclast differentiation[38]. In MMP-9 null mice enchondral bone formation and formation of long bones is disturbed. There is an association between MMP-9 and the release of angiogenic factors[39] from the extracellular matrix. We did not encounter MMP-2 and MMP-9 activity in association with accumulations of inflammatory cells and wear particles.

It indicates that wear particles in the interface tissue do not necessarily lead to activation of macrophages and induction of osteolysis. Wear particles were found to be present extracellularly and intracellularly in interface tissue without evidence for an inflammatory reaction. This is in contrast with current views, based on in vitro studies and the finding of increased levels of mediators of macrophage activation like TNF-alfa and IL-1[6]. However, animal studies[10,40-43] have shown that particles alone do not cause osteolysis, which is consistent with the findings in the present study.

Particle size has been shown to influence the macrophage response[44]. The particles we found in the present study were within the range considered to initiate an inflammatory response. Osteolysis has been shown to be dependent on the concentration of wear particles[45,46]. Although we did not measure the wear on the radiographs, the overall aspect of loosening was one of a radiolucent line completely surrounding the implant, without granulomas and a significant amount of wear.
The present study shows the importance of examining the environment around prosthetic implants and investigating processes in situ, for the elucidation of molecular mechanisms involved in aseptic loosening of hip replacements
The absence of an inflammatory response in the presence of wear particles supports the view that considers fluid-pressure as a more likely cause for osteolysis and loosening[9,10,40-43,47-49,].

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