

# Cathepsin K in Aseptic Hip Prosthesis Loosening: Expression in Osteoclasts Without Polyethylene Wear Particles

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**ABSTRACT. Objective.** To investigate the expression of the bone matrix degrading cysteine proteinase cathepsin K and to determine the colocalization of cathepsin K with polyethylene (PE) particles in tissue specimens of patients with aseptic hip prosthesis loosening (AHPL).

**Methods.** The expression of cathepsin K was studied by immunohistochemistry in tissue specimens of 9 patients with aseptically loosened acetabular components of failed cementless total hip replacements. The expression of cathepsin K was compared to that of the macrophage marker CD68 by serial section analysis. Double labeling of the expression of cathepsin K or CD68 by immunohistochemistry and of PE particles by modified Oil Red staining method was performed.

**Results.** Cathepsin K could be predominantly detected in osteoclasts attached to the bone tissue, while only a few (CD68+) mononuclear and multinucleated foreign body giant cells (MGC) were positive for this enzyme. By double labeling with Oil Red staining we found the majority of CD68 positive cells of the periprosthetic tissue that were colocalized with PE particles. However, cathepsin K-expressing osteoclasts could not be stained with Oil Red.

**Conclusion.** The present data suggest that in AHPL neither mononuclear cells nor MGC but rather osteoclasts are mainly involved in cathepsin K mediated bone matrix destruction. Using double labeling of immunohistochemistry and Oil Red staining we observed that the cathepsin K-expressing osteoclasts did not include PE particles. (J Rheumatol 2001;28:1615–9)

## Key Indexing Terms:

CATHEPSIN K

OSTEOCLASTS

POLYETHYLENE PARTICLES

ASEPTIC HIP PROSTHESIS LOOSENING

The most frequent and important longterm complication of total hip arthroplasty is aseptic hip prosthesis loosening (AHPL), which results in bone loss and implant loosening. AHPL is associated with the presence of a foreign body macrophage response to wear particles of the prosthesis, including metal alloys, ultra high molecular weight polyethylene, and polymethylmethacrylate<sup>1-4</sup>. Among these, particulate wear debris from polyethylene (PE) is considered to play a central role in AHPL. PE laden macrophages were found to express and produce several humoral factors, such as tumor necrosis factor- $\alpha$ , interleukin 1 $\beta$  (IL-1 $\beta$ ), and IL-6, which are known to stimulate osteoclastic bone degradation<sup>3,5,6</sup>. Further, the expression of several matrix-degrading proteinases, including matrix metalloproteinases and cysteine proteinases, was described in macrophages and

multinucleated foreign body giant cells (MGC) that stored PE particles<sup>7-10</sup>.

Since the recent discovery of the cysteine proteinase cathepsin K, a number of biological data have suggested that this enzyme plays a critical role in bone resorption<sup>11-13</sup>. Cathepsin K is predominantly expressed in osteoclasts. Further, it has been shown to degrade the major bone collagen type I and the bone matrix protein osteonectin<sup>12,13</sup>. Deficiency of cathepsin K results in pyknodysostosis, an autosomal recessive osteosclerotic skeletal dysplasia with impaired bone resorption<sup>14</sup>. Most interestingly, cathepsin K is suggested to be unique among mammalian proteinases as it has been found to cleave the collagen type I molecule both inside and outside the helical region<sup>13</sup>.

We investigated the expression of cathepsin K in tissue specimens of patients with aseptically loosened acetabular components of failed cementless total hip replacements. Colocalization with the macrophage marker CD68 was examined by serial section analysis. Moreover, we studied the association of cathepsin K and the PE particles. PE particles are visible under polarized light due to the birefringency of the crystalline components within the polyethylene<sup>1</sup>. In addition, Oil Red, a dye commonly used for tissue cholesteryl esters and triacylglycerols<sup>15</sup>, has been shown to stain PE particles in several studies<sup>16-18</sup>. We exam-

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ined cathepsin K positive cells by polarized light and, further, developed a double labeling method of immunohistochemistry and the Oil Red staining technique.

## MATERIALS AND METHODS

Tissue specimens of acetabular bone implant interfaces were obtained from 9 patients (8 female, one male) during revision surgery of uncemented aseptically loosened total hip replacements of the ABGI type (Anatomique Benoist Girard, Howmedica, Staines, UK). The samples were fixed in 4% phosphate buffered saline-buffered formaldehyde for 24–48 h, processed, and paraffin embedded according to standard methods. Sections 4 µm thick were cut and mounted on silane coated slides. Afterwards, they were dewaxed and rehydrated through gradient alcohols. Immunohistochemical analysis was performed with monoclonal antibodies against human CD68 (clone PG-M1; Dako, Glostrup, Denmark; diluted 1:100) and cathepsin K (clone 182-12G5; Chemicon, Temecula, CA, USA; diluted 1:2000). As control, mouse immunoglobulins (Cymbus Biotechnology, Chandlers Ford, UK; diluted 1:200) were used to replace the primary antibodies. In the case of CD68 labeling, slides were pretreated at 37°C with 0.1% trypsin (Sigma, St. Louis, MO, USA) for 15 min. For single staining, immunohistochemistry was applied using the streptavidin-alkaline phosphatase technique. Slides were first covered with an avidin/biotin blocking kit (Vector, Burlingame, CA, USA) diluted 1:1000 in Tris buffer (pH 7.6). Then nonspecific binding of immunoglobulins was blocked by incubation with 4% nonfat dried bovine milk (Sigma)/2% normal horse serum (Vector) in Tris buffer. Primary antibodies were incubated 1 h followed by 30 min incubation with biotinylated horse anti-mouse IgG (Vector; dilution 1:200 in Tris buffer). Slides were then covered with streptavidin conjugated alkaline phosphatase (Dako) for 30 min in a 1:50 dilution in Tris buffer. The color reaction was performed using the new fuchsin method, endogenous alkaline phosphatase was blocked by adding levamisol (Sigma) to the substrate solution. Finally, slides were counterstained with hematoxylin (Merck, Darmstadt, Germany) for 5 min and mounted with glycerol gelatin (Merck).

In the case of double labeling with the Oil Red staining, expression of CD68 and cathepsin K was first examined by the immunogold-silver technique<sup>19</sup>. Oil Red staining was performed according to a modified method<sup>16</sup>. Oil Red 0.5 g (Oil Red EGN, Sigma) and 100 ml propylene glycol (Sigma) were mixed at maximal 95°C. The solution was then filtered several times. After immunohistochemical analysis, slides were placed in 100% propylene glycol for 5 min. Staining with the Oil Red solution was performed for about 72 h at 40°C. Labeling was then differentiated with 85% propylene glycol and with double distilled water for 5–10 min. Finally, slides were counterstained with hematoxylin and mounted with glycerol gelatin.

Tissue sections were examined and photographed with a Leica DMRX microscope (Leitz, Wetzlar, Germany). Microscopic evaluation of the slides was carried out by a modified method of Hansen, *et al*<sup>19</sup>. Immunohistochemical analysis of all slides was performed on the same day. The following cell types were analyzed separately: mononuclear cells, multinucleated foreign body giant cells, and osteoclasts. The extent of binding was estimated by counting the number of positive cells/5 high power fields. Results were classified according to 4 categories: – no cells positive; (+) less than 20% of cells positive; + between 20% and 50% of cells positive; ++ between 50% and 90% of cells positive; +++ more than 90% positive. Final scores represent the average of scores for all tissue specimens examined.

## RESULTS

Results of the microscopic evaluation of the slides are summarized in Table 1. All specimens contained inflammatory tissue with mononuclear cells and multinucleated foreign body giant cells (MGC) that strongly expressed CD68 (Figure 1). By polarized light, numerous polyethylene

Table 1. Results of microscopic evaluation of cell types.

	OCL	MGC	MC
CK	+++	+	(+)
CD68	+++	+++	++
CK+OR	–	+	(+)
CD68+OR	–	+++	++

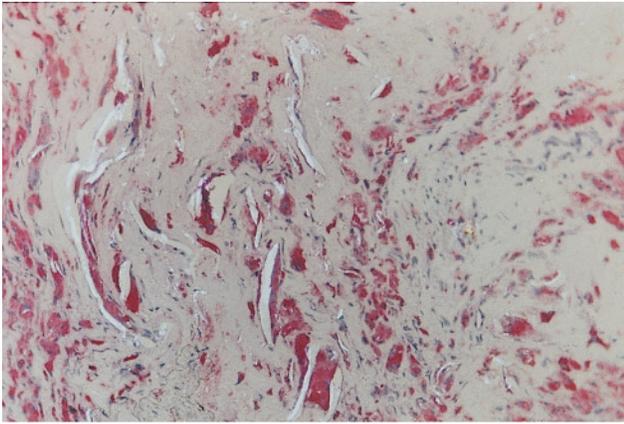
Expression was scored as follows: – no cells positive; (+) < 20% positive cells; + 20–50% positive cells; ++ 50–90% positive cells; +++ > 90% positive cells. CD68+OR: coexpression of CD68 and Oil Red positive cells; CK: cathepsin K; CD+OR: coexpression of cathepsin K and Oil Red positive cells; MC: mononuclear cells; MGC: multinucleated foreign body giant cells; OCL: osteoclasts.

(PE) particles could be observed both intracellularly and extracellularly. CD68 positive cells contained PE particles or surrounded extracellular large PE debris (Figure 1). In contrast, analysis of serial sections displayed only few MGC surrounding large PE debris positive for cathepsin K (Figure 2). The large PE debris revealed intense orange-red color by staining with Oil Red (Figure 3). Oil Red also stained PE laden cells that coexpressed CD68 in a large percentage (Figure 3). Cathepsin K was detected in numerous osteoclasts that were attached to the bone (Figures 4–6). These cells were not colocalized with PE particles by polarized light or by Oil Red staining (Figures 5 and 6). However, these cells were frequently surrounded by Oil Red positive cells (Figure 6). By polarized light, PE particles were observed in the vicinity of the cathepsin K positive osteoclast also (Figure 5). Some of these PE laden cells were also located at the bone surface (Figure 5). Both cells surrounding osteoclasts and osteoclasts attached to the bone expressed CD68 at a very high level (see Table 1). Negative controls did not show positive staining.

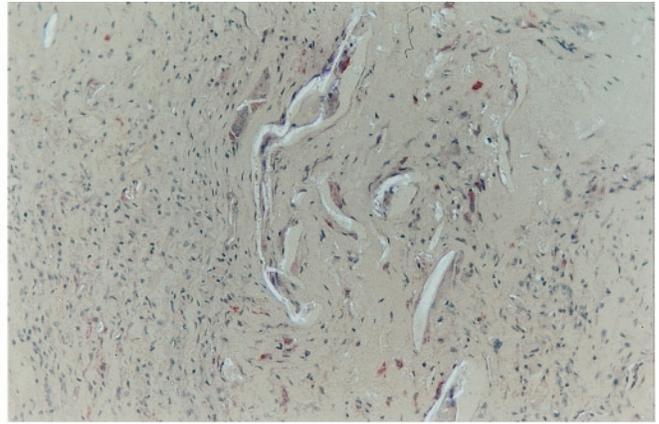
Tissue specimens of 2 patients obtained at the first hip implantation surgery showed strong expression of CD68 in the fibrous capsule and to a lesser extent in the synovium. In contrast, cathepsin K staining was almost absent in these tissues. Negative controls did not show positive staining (data not shown).

## DISCUSSION

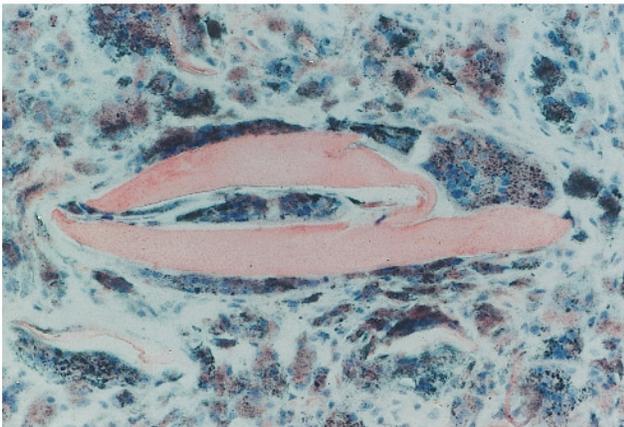
Polyethylene (PE) wear particles are considered to play a central role in aseptic hip prosthesis loosening (AHPL), which is the most important complication of total hip arthroplasty. PE particles are known to induce an inflammatory tissue reaction and cytokine response leading to osteolytic processes<sup>1,3,5,6</sup>. Moreover, several matrix degrading proteinases have been observed in the PE storing cells of the periprosthetic tissues and are suggested to mediate bone destruction<sup>8–10</sup>. Several recent studies reported that the cysteine proteinase cathepsin K is critical for bone resorption<sup>11–13</sup>. Therefore, we investigated the expression of this cysteine proteinase in tissue specimens of patients with



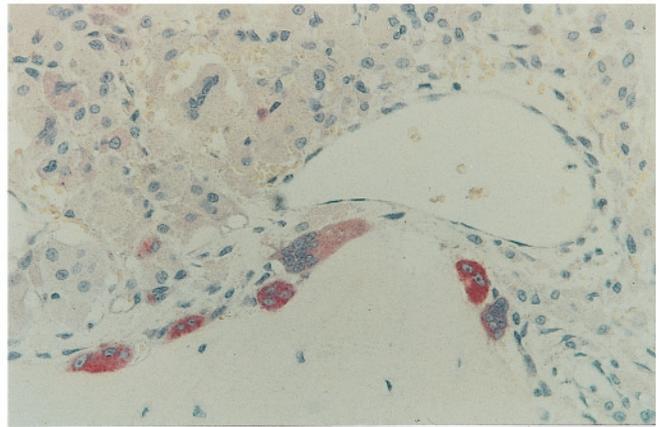
*Figure 1.* Numerous CD68 positive mononuclear cells and multinucleated foreign body giant cells (MGC, red) can be detected in the periprosthetic tissue. Note birefringent PE debris particles surrounded by CD68 positive MGC. Counterstaining with hematoxylin (x200).



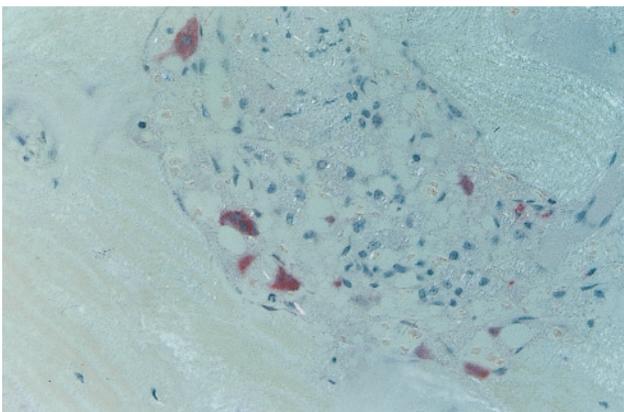
*Figure 2.* Serial section of the slide in Figure 1 reveals few cells expressing cathepsin K (red). Counterstaining with hematoxylin (x200).



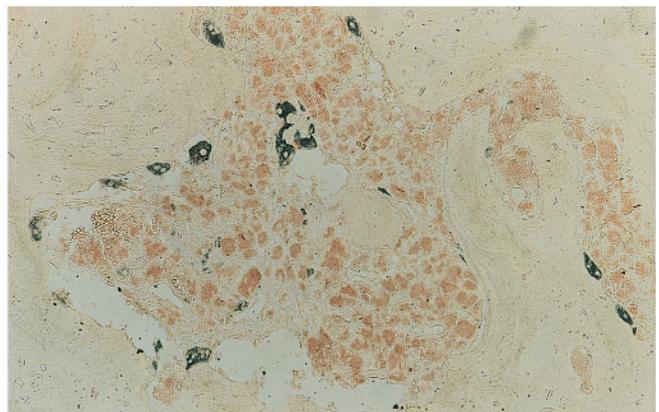
*Figure 3.* Double labeling of CD68 (black) and Oil Red (orange). Numerous CD68 positive cells are also stained by Oil Red. Note 2 large PE particles intensely colored by Oil Red. Counterstaining with hematoxylin (x600).



*Figure 4.* Strong cathepsin K expression (red) in osteoclasts attached to the bone. Counterstaining with hematoxylin (x400).



*Figure 5.* Several cathepsin K-expressing osteoclasts (red). They do not contain PE, but are surrounded by needle-like birefringent PE particles. Counterstaining with hematoxylin (x200).



*Figure 6.* Double labeling of cathepsin K (black) and Oil Red (orange) reveals several cathepsin K-expressing osteoclasts that are negative for Oil Red. Several cathepsin K positive osteoclasts are surrounded by Oil Red positive cells (x200).

aseptically loosened acetabular components of failed cementless total hip replacements.

The expression of cathepsin K could be observed in numerous osteoclasts attached to the bone, whereas only few mononuclear cells and multinucleated foreign body giant cells (MGC) of the periprosthetic tissue were positive for this enzyme. Expression of cathepsin K has been shown in different cell populations such as synovial fibroblasts<sup>20</sup>, macrophages and smooth muscle in atheroma<sup>21</sup>, and in various cell types of the lung<sup>22</sup>. However, several studies indicated that cathepsin K is predominantly expressed in osteoclasts<sup>11,23,24</sup>. This view is confirmed by the present data.

Several osteoclast markers like tartrate resistant acidic phosphatase and the vitronectin receptor (CD51) were detected in macrophages and MGC of periprosthetic tissue<sup>25</sup>. Additionally, the matrix metalloproteinase-9 that is considered to be strongly expressed in osteoclasts<sup>26,27</sup> was observed in MGC and macrophages of patients with AHPL<sup>8</sup>. It was therefore suggested that these cells mediate bone matrix degradation by expressing this osteoclastic proteinase<sup>8</sup>. In contrast, our data contribute to the view that in AHPL it is osteoclasts, rather than mononuclear cells or MGC of the periprosthetic tissue, that are predominantly involved in bone resorption by cathepsin K.

As several studies suggested that PE wear particles could influence the expression of matrix degrading proteinases in AHPL we examined the colocalization of PE particles and the expression of cathepsin K. On standard paraffin embedded tissue specimens, PE particles are visible under polarized light due to their birefringency<sup>1</sup>. However, identification of these particles by polarized light microscopy can be difficult because most of the PE particles are in a size range of about 0.5 to 1.0  $\mu\text{m}$ <sup>1,2,28,29</sup>. Oil Red, a dye commonly used for tissue lipids<sup>15</sup>, was found to detect PE particles in paraffin embedded tissue sections<sup>16-18</sup>. By comparison of light and transmission electron microscopy, it was shown that Oil Red also stained submicron PE particles that are too small to be clearly resolved by light microscopy<sup>16,18</sup>. Commonly performed immunohistochemical examinations of periprosthetic tissue probes may not be optimal as brightness diminishes when both PE particles and protein expression are studied by polarized light. This impairs microscopic examination. To investigate the colocalization of PE particles and the expression of protein molecules under normal microscopic conditions, we developed a double labeling method of immunohistochemistry and a modified Oil Red staining technique. We observed colocalization of Oil Red positive cells and the macrophage marker CD68 in a large percentage (> 90%, compare Table 1) of mononuclear cells and MGC. Our results suggest this double labeling technique is a suitable method to investigate the colocalization of PE particles with specific proteins.

In contrast to the strong staining of CD68 in numerous

Oil Red positive cells, osteoclasts expressing cathepsin K could not be detected for Oil Red reaction. Applying single staining immunohistochemistry and polarized light, cathepsin K positive osteoclasts did not reveal birefringent particles. It is still a matter of debate whether osteoclasts are involved in particle phagocytosis in AHPL. Wang, *et al* demonstrated<sup>30</sup> that osteoclasts derived from human giant cell tumor of bone and from neonatal rat long bones are capable of phagocytosing both polymeric and metallic biomaterial particles. In contrast, previous histological studies reported that osteoclasts *in situ* are not involved in the phagocytosis of wear particles<sup>1</sup>. Our data confirm these studies. We conclude that cathepsin K-expressing osteoclasts do not perform phagocytosis of PE particles. Interestingly, numerous Oil Red positive cells were detected in the vicinity of cathepsin K-expressing osteoclasts. This was evidenced by applying single staining for cathepsin K and polarized light that revealed several birefringent particles surrounding the cathepsin K positive osteoclasts. Several studies presented evidence that the PE laden cells produce osteoclast-stimulating cytokines like IL-1 $\beta$  and IL-6 and tumor necrosis factor- $\alpha$ <sup>3,5,6</sup>. Further investigation should elucidate if the Oil Red positive PE laden cells influence cathepsin K expression of osteoclasts by these cytokine stimuli. Since additionally some of these cells were found on the bone surface, they may be directly involved in bone degradation as well.

In summary, our data indicate that in AHPL the major bone degrading enzyme cathepsin K is predominantly expressed in osteoclasts. It is therefore suggested that neither mononuclear cells nor MGC of the periprosthetic tissue, but rather osteoclasts are involved in cathepsin K mediated bone matrix destruction. Moreover, this is the first report on double labeling of protein molecules by immunohistochemistry and PE particles by modified Oil Red staining. Applying this method, we demonstrated that cathepsin K-expressing osteoclasts do not include PE particles. This contradicts the view that in AHPL osteoclasts are capable of phagocytosing PE particles. It remains to be determined whether PE laden cells surrounding the cathepsin K positive osteoclasts influence the expression of this proteinase by cytokine stimuli.

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