Interleukin-6 in aseptic loosening of total hip replacement prostheses

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Abstract

Objective

To investigate the level of interleukin-6 (IL-6) expression in the synovial-like interface membrane (SLIM) and in the pseudosynovial tissue surrounding the artificial hip joints, as well as in the pseudosynovial fluid from aseptically loosened total hip replacement (THR) prostheses.

Methods

A series of methods were used in this study including immunohistochemical staining, double immunofluorescence labeling, enzyme-linked immunosorbent assay (ELISA), and reverse transcriptase polymerase chain reaction (RT-PCR).

Results

IL-6 was found in all SLIM and the pseudosynovial tissue samples from aseptic loosening of THR. Semi-quantitative morphometry showed that IL-6 containing cells were more numerous in the SLIM (911 ± 197; $p < 0.01$) and the pseudosynovial tissue samples (883 ± 310; $p < 0.01$) than in the control synovial tissue (291 ± 184). Double labeling confirmed that macrophages and fibroblasts were the predominant cell types expressing IL-6. These findings were confirmed by RT-PCR. ELISA revealed no difference in the IL-6 concentration between the pseudosynovial fluid and the control synovial fluid obtained from the patients undergoing hip arthroscopy.

Conclusions

IL-6 locally produced in SLIM may in a paracrine manner contribute to periprosthetic osteolysis of the nearby bone. In contrast, fluid phase IL-6 does not seem to contribute to this end.

Key words
Interleukin-6, total hip replacement, aseptic loosening, interface.

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Introduction

Aseptic loosening is the predominant cause of failure of total hip replacement (THR). Its main symptom is pain and the main finding is periprosthetic resorption of bone as seen in x-rays (1, 2). The mechanisms underlying these processes are thought to be secondary to the host reaction to particulate wear debris (3, 4). Synovial-like interface membrane (SLIM) is invariably found around aseptically loosened THR prostheses (5). Previous studies have shown that macrophages and fibroblasts are the predominant cell types in SLIM (6, 7). These cells can produce a variety of inflammatory mediators that are directly or indirectly involved in the bone resorption and implant loosening (5, 8-12). The cytokines produced in the pseudosynovial tissue can also reach the implant-to-host interface via the synovial fluid. It is so far not clear yet, which of these two tissues is more important from the point of view of aseptic loosening .

Interleukin-6 (IL-6) is a 26 KDa cytokine produced by marrow stromal cells, monocyte-macrophages, myeloma cells, lymphocytes, endothelial cells, fibroblasts and osteoblasts. IL-6 is an autocrine/paracrine factor, and can induce osteoclast formation and bone resorption (13-16). In this study immunohistochemistry, image analysis and RT-PCR were employed to assess the eventual presence, cellular localization and extent of IL-6 expression in SLIM and pseudosynovial tissue from loose THR and to compare it with synovial membranes obtained from primary THR performed for osteoarthritis (OA) of hip. ELISA was used to analyze the level of IL-6 in the pseudosynovial fluid and the synovial fluid collected from hip arthroscopy.

Patients and methods

Patients and samples

The sample-harvesting procedure was approved by the ethical committee of University of Helsinki according to the Declaration of Helsinki. Ten SLIM samples from the prostheses/bone interface in the osteolytic areas and 10 pseudosynovial tissue samples surrounding the artificial hip joints were obtained during revision THR operations performed for aseptic loosening of implants. Of the 10 patients, 6 were women and 4 were men, and their mean age was 72.7 years (44-89). The original disease for primary THR was in all cases OA. The mean interval from primary THR to revision operations was 9.4 years (6-25). For comparison, 10 synovial membrane samples were obtained from patients undergoing primary THR due to hip OA. Of these patients, 6 were women and 4 were men, and their mean age was 56.3 years (38-82). All samples were snap-frozen in isopentane precooled by dry ice and stored at -70°C.

For the ELISA study, 15 pseudosynovial fluid were collected during revision THR. Nine of these patients were women and 6 men, and their mean age was 70.1 years (41-85). For comparison, 10 samples of synovial fluid were obtained at arthroscopy performed to examine painful hip joints. Two of these patients were women and 8 men, and their mean age was 56.3 years (30-82). The synovial fluid samples were centrifuged and stored at -70°.

Immunohistochemistry

The cryostat sections (6 µm thick) were fixed in cold acetone for 5 minutes at +4°C. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in absolute methanol for 30 minutes at +22°C. The sections were then incubated with: 1) normal goat serum [Vector Laboratories, Burlingame, CA; diluted 1:50 in 20 mM Tris-HCl, 150 mM NaCl, pH 7.5 (=TBS)] for 20 minutes at +22°C; 2) polyclonal rabbit antiserum to human IL-6 (Genzyme, MA; diluted 1:100 in TBS containing 0.1% BSA) overnight at +4°C; 3) biotinylated goat anti-rabbit IgG (Vector Laboratories; diluted 1:100 in TBS containing 0.1% BSA) for 30 minutes at +22°C; 4) avidin-biotin -peroxidase complex (Vector Laboratories; diluted 1:200 in TBS) for 30 minutes at +22°C. Finally, the sites of peroxidase binding were revealed with a combination of 3,3'-diaminobenzidine tetrahydrochloride and H_2O_2 (DAB substrate kit, Vector

Laboratories) for 5 minutes at +22°C. Between two steps the sections were washed 3 x 5 minutes in TBS. Half of the sections were counterstained with hematoxylin and then the sections were dehydrated in a gradual ethanol series, cleared in xylene and mounted with Diatex. Omission of antiserum to human IL-6 and use of nonimmune rabbit IgG served as negative staining control.

Image analysis

The average number of IL-6 positive cells was calculated in five different high power fields (x400) by a low light charge-screen coupled CCTV camera (Panasonic WV-CD 130L, Osaka, Japan) mounted on an Olympus BH-2 light microscope linked to a semiautomatic Kontron image analysis and processing systems (Kontron Bildanalyse GMBH, Eching, Germany) equipped with the VIDAS 2.1 program (Kontron Elektronik, Eching, Germany). The number of IL-6 positive cells was calculated in three representative samples in all study groups to get an estimate for the population mean. Using the formula:

$$t = (x - \mu) / SEM$$

it was calculated that the mean of five high power fields is not significantly ($p > 0.05$) different from the population mean (t refers to t-statistics, x = the sample mean, μ = the population mean, SEM = standard error of the mean). Results were expressed as the number of IL-6 positive cells per one mm^2 of tissue. Statistical software of BMDP-PC 7.01 was used to calculate the mean ± SEM to describe the dispersion of the data. Normality of the distributions was checked using the Wilk's W test. The significance of differences between means was analyzed by t-test.

Double immunofluorescence labeling

Cryostat sections were fixed in cold acetone for 20 minutes at -20°C. After washing in PBS, the sections were incubated with the following reagents: 1) monoclonal mouse anti-human 5B5 (IgG_1, a fibroblast marker, DAKO, Glostrup, Denmark; diluted 1:100 in PBS containing 1.25% BSA) or monoclonal mouse anti-human CD68 (IgG_1, a macrophage marker, DAKO; diluted 1:100 in PBS containing 1.25% BSA) for 60 minutes at +22°C; 2) FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA; diluted 1:100 in PBS containing 12.5% BSA) for 45 minutes at +22°C in the dark; 3) polyclonal rabbit antiserum to human IL-6 (Genzyme; diluted 1:200 in PBS containing 1.25% BSA) for 60 minutes at +22°C; 4) TRITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories; diluted 1:100 in PBS containing 12.5% BSA) for 45 minutes at +22°C in the dark. Human IgG (8%; Sigma, St. Louis, MO) was added to the conjugated secondary antibody solutions to reduce nonspecific reaction. The sections were washed with PBS for 3 x 10 minutes between two steps. The sections were air-dried and mounted with Vectashield (Vector Laboratories) and kept in the dark at +4°C.

ELISA

IL-6 concentrations in the pseudosynovial fluid and the synovial samples obtained from hip arthroscopy were measured by ELISA kit (Amersham, London, UK). The microplates (96 wells) were coated with a monoclonal mouse anti-human IL-6 IgG. Duplicate samples of pseudosynovial fluid and control synovial fluid (100 µl per well) were added and incubated for 90 minutes. After washing, a horseradish peroxidase-conjugated goat polyclonal antibody specific for IL-6 was added and incubated for 2 hours. This was followed by the incubation with the substrate solution. Optical density at 450 nm was determined after 30 minutes.

A standard curve was constructed based on the measurements of the known concentrations of an IL-6 standard included on each plate (Research and Diagnostic Systems Inc., Minneapolis, MN). The results were not accepted if the correlation coefficient of the standard curve was less than 0.98. In most of the kits it was measured as 0.99-1.00. Within-assay precision was 2.7-4.2% and between-assay precision was 2.4-7.8% in different dilutions. The minimum detectable dose of IL-6 was 0.35 pg/ml.

RT-PCR

The cryostat sections (6 µm) were prepared for mRNA extraction using oligo $(dT)_{25}$ covalently attached to magnetic polystyrene microbeads via 5' linker group from Dynabeads mRNA DIRECT kit (Dynal, Oslo, Norway). Extraction was performed according to manufacturer's instruction.

The reverse transcription (Perkin Elmer, Branchburg, NJ) reaction was performed using 5.0 U of the enzyme in a total volume of 20 µl (90 mM KCl, 10 mM Tris-HCl, pH 8.3, 1 mM $MgCl_2$, 200 mM of dATP, dCTP, dGTP and dTTP) in PCR tubes topped with 50 ml mineral oil (Sigma) on a thermal cycler (Pharmacia, Sollentuna, Sweden). The reaction was run for 1 minute at +22°C, 1 minute at +37°C, 5 minutes at +55°C and 10 minutes at +70°C.

Second strand synthesis was performed in PCR buffer containing sense primer (0.17 mM) and 100 mM of dATP, dCTP, dGTP and dTTP in a total volume of 30 µl topped with 50 µl of mineral oil. The thermostable DNA polymerase (0.5 U, Finnzymes, Espoo, Finland) was added, and the samples were denatured at +95°C for 2 minutes and annealed at +61°C for 1 minute followed by 5 minute extension at +72°C and 2 minutes denaturation at +95°C. Second strand cDNA supernatant was transferred to a new PCR tube, where the antisense primer (0.17 µM) was pipetted and for amplification 45 cycles of 1 minute at +95°C, 1 minute at +61°C and 1 minute at +72°C were performed. Target specific primers for sense (5'- TCC AGT TGC CTT CTC CCT GG-3') and for antisense (5'-TCT GCC AGT GCC TCT TTG CT-3') with a 231 bp long product were used for amplification. Amplified DNA was run on a 1% modified agarose gel (FMC Bioproducts, ME) for size verification. For a negative control PCR was done without template and also without both template and primers. mRNA extraction was controlled using primers for -actin.

Nucleotide sequencing of the PCR

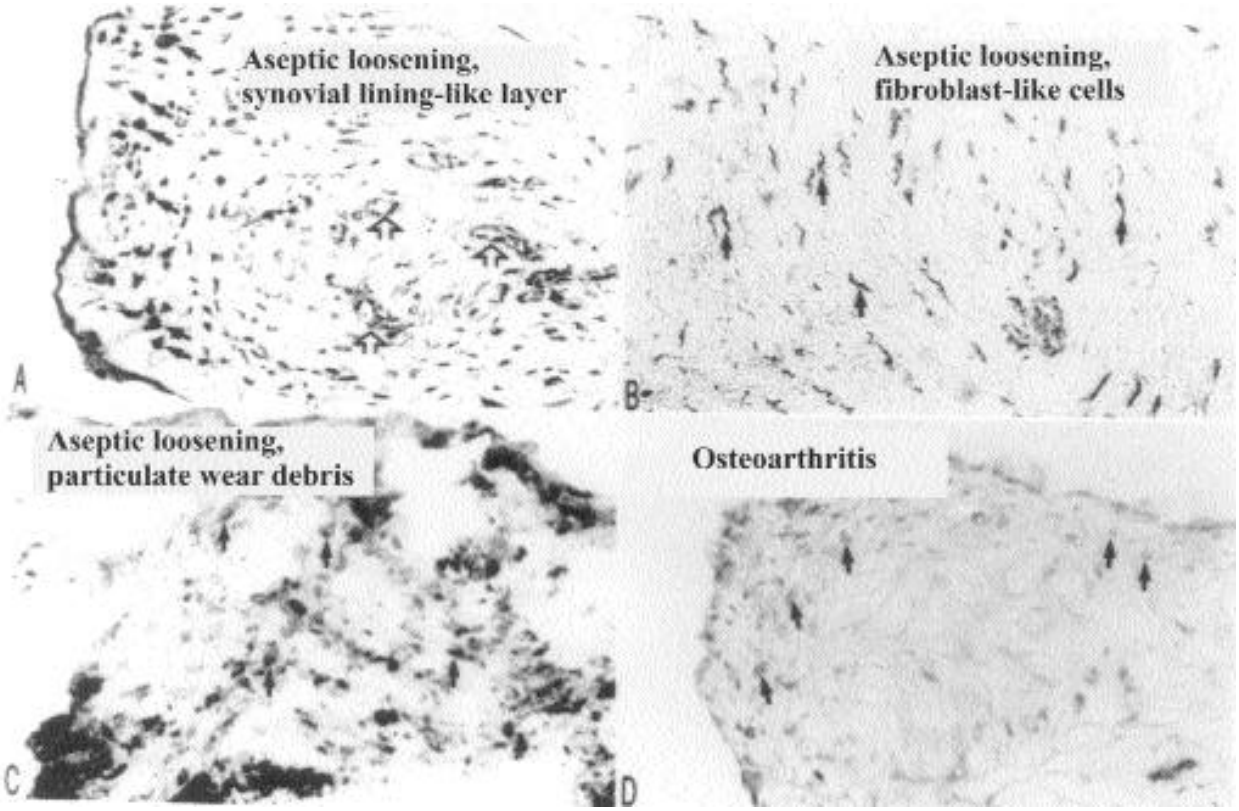

Fig. 1. (**A**) Interleukin-6 (IL-6) immunoreactive cells in the lining-like layer (**black arrows**) and vascular endothelial cells (**open arrows**) in the synovial membrane-like interface membrane (SLIM) in a patient with aseptic loosening of the total hip replacement (THR) implants. (**B**) IL-6 immunoreactive fibroblast-like cells (**arrows**) embedded in interstitial collagen fiber-rich matrix in SLIM in a patient with aseptic loosening of the THR implants. (**C**) Particulate wear debris phagocytozed by macrophage-like cells (arrows) and embedded in the extracellular matrix in SLIM in a patient with aseptic loosening of the THR implants. (**D**) IL-6 positive cells (**black arrows**) in a control osteoarthritic synovial membrane sample.

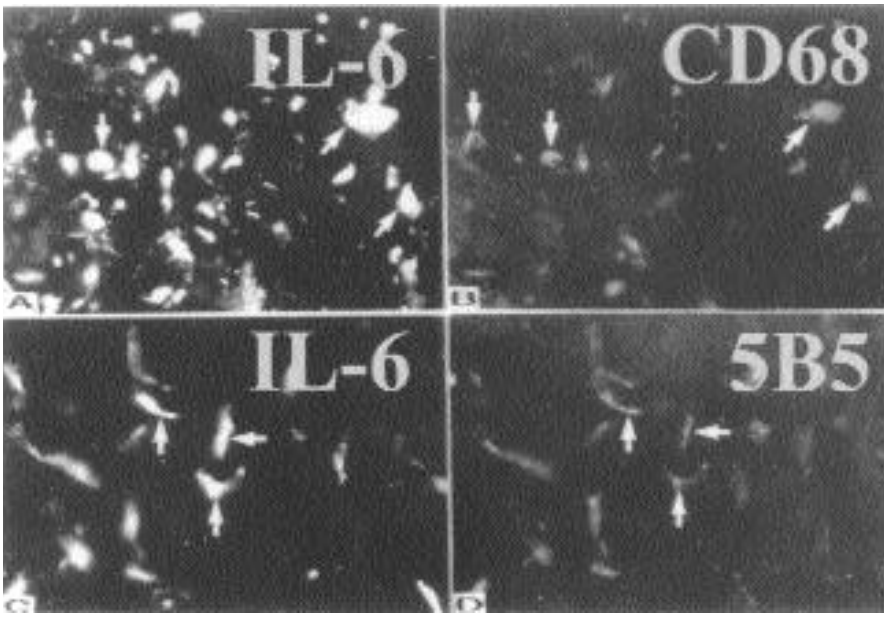

Fig. 2. Double immunofluorescence labeling of interleukin-6 (IL-6) and macrophages (CD68 staining) or fibroblasts (5B5 staining). Panels A and B were photographed from the same field of a synovial membrane-like interface membrane (SLIM) sample: (**A**) IL-6 labeling (**white arrows**) combined with (**B**) CD68 labeling showing that some of the IL-6 immunoreactive cells are CD68-positive macrophages (**white arrows**). Panels C and D were photographed from the same field of a SLIM sample: (**C**) IL-6 labeling (**white arrows**) combined with (**D**) 5B5 labeling showing that some of the IL-6 immunoreactive cells are 5B5-positive fibroblasts (**white arrows**).

amplification products was used to confirm the identity of the amplified bands. PCR fragments were extracted from the gel using silica-gel membrane-based QIAquick columns according to the manufacturer's protocol (Qiagen Inc., Chatsworth, CA), quantified, sequenced (20-75 ng/PCR fragment) using fluorescein-labeled dye terminator kits supplied by ABI (PE Applied Biosystems, Foster City, CA) and analyzed on Applied Biosystems automatic sequencer 373 A (PE Applied Biosystems).

Results

Morphological evaluation and cell-typing

The SLIM and pseudosynovial tissue samples were histologically similar and both of them possessed the synovial lining-like layers, usually one to three layers thick. They consisted of highly cellular areas containing macrophage and fibroblast-like cells, and vascular endothelial cells and fibrous tissue con-

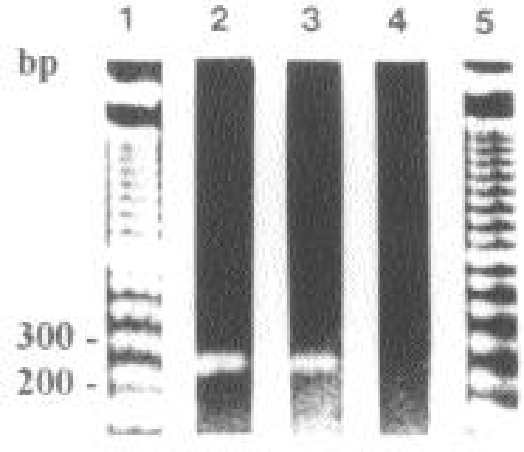

Fig. 3. Reverse-transcriptase-polymerase chain reaction (RT-PCR): Lanes 1 and 5:100 base pair standard ladders; lane 2: the positive IL-6 sample control and lane 3: amplified DNA products of interleukin-6 mRNA from a synovial membrane-like interface membrane show a 231 base pair long amplicon; lane 4: a negative RT-PCR control with primers only.

sisting mainly of relatively dense collagenous fibers and elongated fibroblasts. In many areas the implant-derived debris was seen in the SLIM and pseudosynovial tissue samples. Metal particles were identified as irregularly shaped, fine black particles. Small metal particles were often phagocytosed by macrophages which were IL-6 positive, but large particulate debris were frequently seen in the extracellular matrix. Polyethylene debris were abundant and identified as highly birefringent fragments under polarized light, and these particles were often phagocytosed by IL-6 positive macrophages.

Immunohistochemistry

In the SLIM and pseudosynovial tissue samples, IL-6 was found in vascular endothelial cells, interstitial fibroblasts and, in particular, in macrophages (Fig. 1A). Occasionally, the IL-6 positive fibroblast-like cells were seen more evenly scattered in the stroma (Fig. 1B). IL-6 positive macrophage-like cells seemed to be located predominantly in the cell-rich areas, which coincided with the implant-derived debris (Fig. 1C). Double immunofluorecence labeling confirmed that macrophages (Fig. 2A, 2B) and to a lesser extent, fibroblasts (Fig. 2C, 2D) were the main IL-6 positive cells. IL-6 appeared in similar cell types also in control OA synovial membrane samples, but staining was relatively weak. The numbers of IL-6 positive cells were higher both in the SLIM (911 ± 197; $p < 0.01$) and pseudosynovial tissue samples (883 ± 310; $p < 0.01$) than in the control OA synovial membrane samples (291 ± 184), while statistical analysis showed no significant difference between the means of the positive cells in the SLIM and pseudosynovial tissue samples. Negative staining control confirmed the specificity of the method.

ELISA

IL-6 level was 774 ± 645 pg/ml in the pseudosynovial fluid samples from aseptic loosening of THR and 17700 ± 54570 pg/ml in hip fluid samples obtained from hip arthroscopy. There is no difference in the level of IL-6 between these samples ($p = 1.21$).

RT-PCR

RT-PCR analysis disclosed the presence of IL-6 mRNA in all samples obtained from revision THR operations. In control OA synovial membrane samples the IL-6 signal was usually relatively weak except in those samples, which showed histological signs of inflammation in the synovial membrane. All experiments were run at least twice with consistent results and the identity of the PCR product was certified using nucleotide sequencing (Fig. 3).

Discussion

Mechanisms leading to aseptic loosening of THR are still not completely understood, although it is obvious that such knowledge could provide a rational basis for prevention of periprosthetic osteolysis. Our hypothesis is that periprosthetic bone loss is caused by a local imbalance between cytokines which stimulate bone resorption and cytokines which stimulate bone formation, and that this periprosthetic bone resorption combined with cyclic mechanical loading lead to aseptic loosening of the implant. Host response to implant materials/debris stimulates macrophages and fibroblasts to produce mediators, which stimulate osteoclasts directly or via interactions with osteoblasts and result in bone resorption. This hypothesis has been supported by recent research (8, 9, 17). The histologic, biochemical and immunohistologic studies have demonstrated that the IL-6 activity levels were significantly higher in interface membranes of failed THRs with femoral osteolysis compared to those without femoral osteolysis. Furthermore, numerous IL-6 positive macrophages, fibroblasts and endothelial cells were present in interface membranes of failed THRs (18).

The current results extend previous work on the part played by cytokines in aseptic loosening by showing the presence of IL-6 positive cells in the SLIM and pseudosynovial tissue samples. Because of ethical reason, it was not possible to obtain corresponding control samples from well-fixed THRs. Thus, the synovial membrane samples from OA were used as the controls in this study. Even so, the quantitative comparison showed that the number of IL-6 positive cells in the SLIM and pseudosynovial tissue samples was higher than in OA synovial samples. The immunohistochemical findings were confirmed by RT-PCR, the pattern of mRNA expression paralleled that of immunoreactivity for IL-6. The current results confirm our hypothesis that cytokines capable of inducing bone resorption are present in elevated concentrations in aseptic loosening of THR although the high age of the patients with aseptic loosening of the THR implants compared to OA controls may have contributed to this difference as

IL-6 production in response to various stimuli seems to increase upon aging (19, 20).

Inflammation may also occur in osteoarthritis and this is accompanied by expression of IL-6. Nevertheless, bone resorption is not a characteristic feature of osteoarthritis. This may relate to the fact that in IL-6 in a normal joint is not produced in close apposition to bone surface as it is in the aseptic loosening of THR implants. In the aseptic loosening IL-6 is produced in synovial membrane-like interface membrane, which is a thin layer of tissue located between the implant and host bone.

The pseudosynovial tissue surrounding the loose artificial hip joints secretes the pseudosynovial fluid and various mediators. The interest in the role of pseudosynovial fluid in the pathogenesis of aseptic loosening is not a novel one and has also been studied by other investigators. We have shown significant presence of IL-6 in pseudosynovial tissues in failed THR. The pseudosynovial fluid containing IL-6 can flow into the interface between prosthesis and bone and may synergistically work with IL-6 produced in SLIM for bone resorption. However, we failed to show any significant difference between the levels of IL-6 in the pseudosynovial fluid and the synovial fluid obtained from hip arthroscopy. This may in part relate to methodological and demographic difference.

Cytokines have important effects on normal and diseased bone as the regulators of cell behaviors (21-23). IL-6 is a multifunctional cytokine which has important local and systemic physiological effects (24, 25). These results are consistent with the IL-6 production by SLIM contributing to local osteolysis and aseptic loosening of THR. Future research addressing to these biochemical processes may reveal ways to inhibit production of bone resorption-inducing factors by macrophages and possibly fibroblasts. Also, materials and prosthetic designs may be improved to reduce the formation of wear particles that trigger these cellular and biochemical processes in the formation of the particulate disease in aseptic loosening of THR.

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