Expression of parathyroid hormone related protein in the tissue around loosened hip prostheses

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Abstract

Objective
To investigate the eventual presence and cellular source of parathyroid hormone related protein (PTHrP) in the synovial-like interface membrane from aseptic loosening of total hip replacement (THR).

Methods
A polyclonal rabbit antiserum to the amino-terminal peptide of human PTHrP was used to stain 10 interface membrane samples from loose THR and 10 synovial tissue samples from hip osteoarthritis (OA). Quantitative microscopic assessment was done with a computer-assisted image analysis system. Western blotting was applied to verify the presence of PTHrP in both tissue samples. Double immunofluorescence labelling aimed to reveal the cellular sources of PTHrP.

Results
Immunoreactive PTHrP was found in all interface membrane and OA synovial tissue samples. The number of PTHrP positive cells in interface membrane was much higher than in OA synovial tissue. Positive cells were most commonly seen in the lining-like layers and sublining area of interface membrane. Double immunofluorescence labelling showed that most macrophages and fibroblasts in interface membrane were PTHrP positive. Western blotting revealed the 24-25 KD bands in both tissue samples.

Conclusions
PTHrP expression is upregulated in interface membrane around loosened hip prostheses. Locally accumulated PTHrP may contribute to periprosthetic osteolysis and aseptic loosening of THR through its direct effects on bone, or indirectly via the induction of inflammatory mediators.

Key words
Parathyroid hormone related protein, total hip replacement, aseptic loosening, interface membrane.

Introduction

About one million total hip replacements (THR) are done worldwide annually. It is the most successful and cost-effective treatment for the disabling end-stage hip diseases. As a result of continuous improvements in surgical techniques, biomaterial research, and implant designs, early and late complications have been greatly reduced. Aseptic loosening of the prosthetic components, however, remains the major long-term complication of THR, accounting for more than 70% of indications for revision operations (1). Formation of a synovial-like interface membrane between the prostheses and bone can often be seen in aseptic loosening of THR. It shares some of the histologic features of both foreign body reaction and chronic synovitis, and plays a pivotal role in peri prosthetic osteolysis (2, 3).

Parathyroid hormone related protein (PTHrP) was first isolated from tumors associated with malignant hypercalcemia. It is a 139- to 173-amino-acid protein with an amino (N)-terminal homology to parathyroid hormone (PTH). PTHrP is expressed in surprisingly diverse tissues (4). Macrophages, fibroblasts, vascular endothelial cells, and osteoblasts can synthesize PTHrP (5-8). While PTH is secreted only via a regulated pathway, PTHrP is secreted through both a regulated and a constitutive pathway in a cell-specific fashion (9). PTHrP acts locally in an autocrine or paracrine fashion more analogous to cytokines than to classic hormones. Compared to PTH, the distribution of PTHrP is much more diffuse, and its function more diverse (10). At present, at least three major secretory forms of the peptide with different biological actions have been found: the N-terminal species, the mid-region species, and the carboxyl (C)-terminal species. The N-terminal peptide is a mature, fully active form of PTHrP. It is well documented that the PTH-like action of PTHrP, such as the stimulation of bone resorption, is confined to its N-terminal region. The mid-region is intensely conserved, but its function is not well established (4). The experiments addressing the eventual role of the C-terminal in osteoclast formation and recruitment have produced conflicting results (11-13). The N-terminal region of PTHrP displays a high-affinity receptor binding and efficient receptor activation. PTHrP and PTH share a common receptor, the type-1 PTH receptor, which exists in the skeletal and renal tissues (14, 15).

We studied the expression of the N-terminal PTHrP in interface membrane from aseptic loosening of THR, and compared this with synovial tissue samples from the patients with hip osteoarthritis (OA) undergoing primary THR.

Patients and methods

Patients and samples

The sample-harvesting procedure was approved by the ethical committee of the University of Helsinki according to the Declaration of Helsinki. Ten interface membrane samples were collected between the prostheses/cement and bone in the osteolysis areas from patients undergoing revision THR due to aseptic loosening of cemented prostheses. Of these patients, 4 were men and 6 women, mean age 67.9 years (range 60-82). Indication for the primary THR was OA. The mean interval from primary THR to revision was 11.7 years (range 5-22). There were no clinical, laboratory, and roentgenographic signs of infection. Ten synovial tissue samples were obtained from patients undergoing primary THR due to OA. Of these patients, 7 were women and 3 men, mean age 71.1 years (range 41-84). These samples were embedded in OCT compound and snap-frozen in isopentane precooled by dry ice and kept at -70°C. Four serial cryostat sections (6 μm thick) were cut from each sample. The first and last sections were stained with hematoxylin. Sections number 2 were used for immunostaining, and number 3 for negative staining control.

Immunohistochemistry

The sections were fixed in cold acetone for 15 minutes at -20°C. Endogenous peroxidase activity was blocked with 0.3% H2O2 in absolute methanol for 30 minutes. The sections were then incu-
Double immunofluorescence labelling
After fixation in cold acetone at -20°C for 20 min, the sections were incubated with the following primary and conjugated antibodies at room temperature: 1) polyclonal rabbit antisera to the N-terminal of human PTHrP (diluted 1:200 in PBS containing 1.25% BSA) for 60 min; 2) TRITC-conjugated goat anti-rabbit IgG (diluted 1:100 in PBS containing 12.5% BSA, Jackson Immunoresearch Laboratories, West Grove, PA) for 45 min; 3) mouse MAb to human CD68 and (a macrophage marker, diluted 1:100 in PBS containing 1.25% BSA, Dako, Glostrup, Denmark), or mouse MAb 5B5 (a fibroblast marker, diluted 1:100 in PBS containing 1.25% BSA, Dako) for 60 min; and 4) FITC-conjugated donkey anti-mouse IgG (diluted 1:100 in PBS containing 12.5% BSA, Jackson) for 45 min. For the negative controls, normal mouse IgG1 and rabbit IgG were used instead of the first and second primary antibodies, respectively.

Human IgG (0.08%, Sigma) was added to conjugated antibody solutions to reduce non-specific staining. Sections were washed with PBS for 3 x 5 minutes between steps. Slides were air-dried and mounted with Vectorshield (Vector Laboratories). Specimens were viewed through a Leitz Aristoplan fluorescence microscope equipped with epi-illumination and appropriate specific filters. The photographs were taken on Kodak T-max film (Rochester, NY) using an automatic Leitz Vario-Orthomat microscope camera.

Western blotting
Tissue samples were minced into small pieces and homogenized in RIPA buffer (Boehringer Mannheim GmbH, Germany) with Ultra-Turrax T25 (Jancy and Kunkle, IKA Laborateknik, Staufen, Germany) in an ice bath. After mechanical homogenization, the samples were further treated with an ultrasonicator Vibra-Cell 501 (Sonics & Materials, Danbury, CT). The samples of homogenized fluid were then centrifuged at 30,000 g for 1 hr at +4°C. Tissue extracts were adjusted to 30 g of total protein per sample in Laemmli sample buffer. Before being applied to the gel, the samples were boiled for 5 min. After electrophoresis, the gel was blotted onto a polyvinylidene membrane (Millipore, Bradford, MA). The membrane was blocked with 3% BSA at room temperature overnight, and was then incubated with polyclonal rabbit antisera to the N-terminal of human PTHrP diluted 1: 800 in antibody dilution buffer (TBS containing 2% BSA and 0.1% Tween-20). After washing with TBS containing 0.1% Tween-20 (TTBS), the membrane was incubated with alkaline phosphatase conjugated anti-rabbit IgG made in goat (diluted 1:5000 in antibody dilution buffer, Silenus Laboratories, Hawthorn, Victoria, Australia) for 1 hr. The membrane was then washed with TTBS for 3 x 10 min. The alkaline phosphatase-binding sites were revealed in color development solution (Alkaline phosphatase conjugated substrate kit, Bio-Rad Laboratories, Richmond, CA) for 10-30 min. The color reaction was stopped by washing the membrane in distilled water for 10 min.

Results
Histological features of interface membrane
Tissue structure and cellularity were examined after hematoxylin staining. The interface membrane was characterized by a foreign body reaction. A continuous lining-like structure was found in 8 samples, while in another 2 cases the lining was interrupted by fibrous tissue. Wear particles from THR components were present in the interface membrane samples. Metal debris appeared as small, black particles. Polymethylmethacrylate bone cement particles could only be observed indirectly in the form of large irregular empty spaces, as they had been dissolved during the sample processing. Wear particles of ultra-high molecular weight polyethylene from the acetabular components were usually not visible under regular light microscopy, but were clearly seen and strongly birefringent under polarized light.

Immunoreactivity of the N-terminal of PTHrP
All interface membrane and OA sam-
samples showed immunoreactive PTHrP. The staining pattern was similar in these tissues, but the number of the positive cells was much higher in interface membrane than in OA synovia. ANOVA demonstrated no statistically significant differences between the results reported by different researchers (p = 0.16). There was a statistical difference (p < 0.0005) between the IM and OA synovial samples (Table 1). PTHrP positive cells were most commonly seen in the lining-like layer and the sublining areas of interface membrane from aseptic loosening of THR. Immunoreactive PTHrP was also detected in macrophage-like cells, endothelial cells and foreign body-type giant cells in the stroma of interface membrane (Fig. 1). In OA synovial tissue samples, PTHrP reactivity appeared in similar areas, but the number of positive cells was much lower. Negative staining control confirmed the specificity of the staining.

Double immunofluorescence labelling for PTHrP and macrophage/fibroblast markers demonstrated that many macrophages in the interface membrane were PTHrP positive. Double positive cells were detected in both the lining-like layer and the sublining area, as well as in the stroma. In contrast, only a small number of macrophages exhibited clear PTHrP immunoreactivity in OA synovial tissue samples (Fig. 2). Similarly, PTHrP reactivity was clearly visible in many fibroblasts in the lining-like layer and sublining area, as well as in the stroma of the interface membrane. In OA synovial tissue samples only a small fraction of fibroblasts exhibited a clear immunoreactive PTHrP (Fig. 3).

Western blotting analysis revealed 24-25 KD bands both in interface membrane around loosened hip prostheses and in OA synovial tissue samples (Fig. 4).

**Discussion**

Our study demonstrates that the number of the PTHrP positive cells was higher in interface membrane from aseptic loosening of THR than that in OA synovial tissue samples. Most of the macrophages and fibroblasts exhibited immunoreactivity in (A) lining cells (x 250), (B) endothelial cells (x 400), (C) macrophage-like cells (x 400), and (D) multinucleate giant cells (x 400) of synovial-like interface membrane. (E) Positive cells in OA synovial tissue (x 250), (F) Negative staining control confirms the specificity of staining (x 250).

![Figure 1](image1.png)

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Table 1. The number of PTHrP-positive cells per 1000 hematoxylin-stained cells.
PTHrP is now recognized as a locally active, bone-resorbing cytokine, and its effects on the skeletal tissues are mediated via the N-terminal sequence. The target cells of PTHrP are osteoblast/stromal cells (20, 21). After binding to PTHrP, osteoblasts can produce osteoclast-activating factors such as macrophage-colony stimulating factor (M-CSF), and play a critical role in osteoclast formation through cell-cell interaction with osteoclast progenitors (22-26). In a mouse model, the N-terminal peptide resulted in a dose-dependent stimulation of tartrate-resistant acid phosphatase-positive multinucleated cell formation. Activation of PKA and PKC is directly linked to PTHrP-stimulated osteoclastogenesis from hematopoietic blast cells (27). PTHrP increases the number of more committed osteoclast progenitors, but does not affect the number of less committed precursors. It acts on mature osteoclasts present in bone marrow (28). A recent study showed that osteoclasts also express PTHrP. It was detected in the majority of actively resoring osteoclasts in sections of mouse long bones. In an intramembranous bone formation model, PTHrP mRNA and protein appeared in the osteoclasts at active bone resorption sites as well as in active osteoblasts and bone lining cells. Both mRNA and protein for PTHrP were also found in the osteoclast-like cells from human giant cell tumours of bone, and in the osteoclasts in sections of bone or joints from patients with the Paget’s disease, rheumatoid arthritis, and OA (29). These results suggest that PTHrP may also affect osteoclast function via an autocrine pathway.

PTHRP is an important inflammatory mediator and may be involved in the complex cytokine network. Expression of PTHrP is upregulated in arthritic synovium. It plays a role in the pathogenesis of arthritis by acting locally in a paracrine fashion in synovial tissue, as well as by acting directly on adjacent cartilage and bone (30). High levels of PTHrP have been found in the synovial fluid of different arthritides, and it is closely related to disease activity (31,32). Synovial fibroblasts can produce PTHrP, and some cytokines such as IL-1 and TNF-α can increase its production (6, 30). PTHrP can, in turn, modulate local synthesis of some cytokines such as IL-6 (30,33). PTHrP induction occurs at a later stage in the cytokine cascade, distal to TNF-α and IL-1, but proximal to IL-6 (30). In addition, PTHrP induces the production of nitric oxide, prostaglandin E2 and matrix metalloproteinases (34-36). Through its role in the inflammation, PTHrP may contribute to periarticular bone destruction.

A major limitation for drawing conclusions from the analysis of interface membrane from aseptic loosening of THR is the difficulty of obtaining appropriate control samples. Theoretically, interface membrane from well-fixed THR could serve as a control.
In conclusion, the expression of PTHrP is upregulated in interface membrane from loosened THR compared to that in OA synovial tissue samples. PTHrP may be involved in aseptic loosening of THR by inducing osteoclastic bone resorption and by affecting the inflammatory cascade.

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