Localization of RANKL in osteolytic tissue around a loosened joint prosthesis

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Abstract Osteoclastogenesis is a key event of the cellular reaction in prosthetic loosening. Immunohistochemistry and reverse transcription-polymerase chain reaction (RT-PCR) were used to study the localization and expression of receptor activator of nuclear factor kappa B ligand (RANKL), a potent factor for osteoclastogenesis in the membranous tissue formed around loosened prosthetic joint implants. RANKL was identified in a wide variety of cells appearing in this membranous tissue. At least three types of RANKL-positive cells were identified, including prolyl 4-hydroxylase (PH)-positive fibroblast lineage cells, CD68 cells, and tartrate-resistant acid phosphatase (TRAP)-positive mononuclear and multinucleated macrophage lineage cells. Tumor necrosis factor (TNF)-alpha-converting enzyme (TACE) was colocalized with RANKL in these cells, suggesting the in-situ release of this factor. RT-PCR confirmed the actual expression of the RANKL and TACE genes in the tissues around the loosened implant. These observational findings indicate the possible synthesis of RANKL by fibroblast and macrophage lineage cells, and suggest the in-situ involvement of RANKL in both osteoclastogenesis and osteoclastic bone resorptive events occurring in prosthetic joint loosening.

Key words loosening · RANKL · TACE

Introduction

The mechanism of cellular reaction to artificially implanted materials is a major concern for orthopedic surgeons. Cellular events occurring around implanted materials sometimes cause aseptic loosening of the implant, leading to serious clinical problems [1,2]. Osteolysis caused by osteoclastic bone resorption is reportedly a key event in the mechanism of loosening, and several factors have been demonstrated to be involved in this mechanism [3–6].

Recently, a potent molecule responsible for osteoclastogenesis, has been identified as a ligand for receptor activation of nuclear factor (NF)-kB (RANKL) [7–9]. RANKL is a membrane-bound 40- to 45-kDa protein that is a member of the tumor necrosis factor (TNF) family. Together with macrophage colony-stimulating factor (M-CSF), RANKL has been shown to support the differentiation and maturation of osteoclasts in vitro in mouse, rat, and human cells. Mice with a disrupted RANKL gene exhibited an osteopetrotic phenotype, suggesting that RANKL plays an essential role in osteoclastic bone resorption [9].

Based on this knowledge, it appears that RANKL may be a candidate molecule responsible for the cellular events leading to prosthesis loosening. RANKL appears to be expressed and produced by cells derived from membranous tissues located adjacent to loosened implants, suggesting that RANKL may be a targeting molecule that regulates the osteolytic events occurring in prosthetic loosening. However, little is known about either the in-situ localization of RANKL or the cell types expressing RANKL in human prosthetic loosening.

The purpose of this study was to elucidate the involvement of RANKL in the cellular mechanism of the osteolytic reaction occurring in the loosened prosthesis. The expression and localization of RANKL in the tissue surrounding the loosened prosthesis were examined by reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry, respectively. In addition, the expression and localization of TNF-alpha converting enzyme (TACE) (shown to cleave RANKL and release its soluble form) were also examined.
Patients and methods

Patients

Tissue samples were obtained during surgery from five patients who underwent revision total hip arthroplasty. Written informed consent was obtained from all patients. All samples were taken from the membranous tissue formed around loosened prosthetic joint implants. In all patients, radiographic and clinical findings indicated aseptic loosening with osteolysis. Occult pyogenic infection was excluded by general clinical examination, full blood count, C-reactive protein levels, and intraoperative macroscopic findings. Duration of implant (i.e., the time from primary surgery to revision surgery) ranged from 6 to 32 years. A summary of patient information is presented in Table 1.

Preparation of tissues

Tissue samples were prepared as previously described [10]. They were fixed in 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany) in phosphate-buffered saline (PBS; pH 7.4) (Sigma Chemical, St. Louis, MO, USA), dehydrated in an ethanol series, and embedded in paraffin. Sections 5-μm were made on a microtome, and some sections were stained with hematoxylin and eosin. The remaining serial sections were prepared for immunohistochemistry.

Immunohistochemistry

Immunohistochemistry was performed using the streptavidin-peroxidase method, with histofine SAB-PO kits (Nichirei, Tokyo, Japan) according to the method recommended by the manufacturer [11]. Three different antibodies were used as primary antibodies: (1) mouse polyclonal antibody against human CD68 (purchased from Dako, Santa Barbara, CA, USA; 1:200 dilution); (2) goat polyclonal antibody against human RANKL (purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:200 dilution); and (3) mouse monoclonal antibody against human prolyl 4-hydroxylase (PH; purchased from Daiichi Fine Chemical, Tokyo, Japan; 1:400 dilution); we also used a rabbit polyclonal antibody against human TACE (purchased from R&D Systems, Minnesota, MN, USA). Tissue sections were briefly deparaffinized, dehydrated, and placed in 3% H₂O₂ in methanol to block endogenous peroxidase. After a washing in PBS (pH 7.2), the sections were blocked with 10% normal serum of the same species as the secondary antibody to minimize background staining, followed by incubation with the primary antibody for 2 h at room temperature. Normal serum of the same species as the primary antibody was used as a control for the primary antibody. After a washing in PBS, the sections were incubated with the secondary antibody (rabbit Ig-G; Nichirei, Tokyo, Japan) for 20 min at room temperature in a humid chamber, and then incubated with peroxidase-conjugated streptavidin (Nichirei) for 20 min at room temperature in a humid chamber and washed in PBS. Finally, color reaction was performed using the substrate reagent 3,3′ diaminobenzidine tetrahydrochloride (Dojindo, Tokyo, Japan). Sections were counterstained with hematoxylin and mounted.

Tartrate-resistant acid phosphatase (TRAP) staining was performed, using a TRAP staining kit (Sigma, St. Louis, MO, USA). TRAP activity was detected according to a procedure using naphthol AS-TR phosphate containing 10 mM L(+)-tartaric acid as substrate. These sections were also counterstained with hematoxylin.

RNA extraction and RT-PCR

Total RNA was extracted from the fibrous tissues of the five patients by an acid guanidine thiocyanate-phenol-chloroform (AGPC) method, using Trizol (Gibco, Grand Island, NY, USA) according to the manufacturer’s instructions. In 1 μg of tRNA from each sample genomic DNA was eliminated with DNaseI (Takara, Japan) and tRNA was reverse transcribed in 20 μl of a reaction mixture containing 200 U of SuperScript II reverse transcriptase (Gibco) and 0.5 μg Oligo(dT)₁₂₋₁₈ primer (Gibco). Subsequently, 1 μl of each reaction product was amplified in 25 μl of a PCR mixture containing 0.125 U of Taq DNA polymerase and 12.5 pmol each of primers (sense and antisense). Oligonucleotides

<table>
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<th>Case no.</th>
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