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Original article

Gene expression of bone-resorbing cytokines in rat osteolysis model

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Abstract Osteolysis after single-injection polyethylene (PE) administration has been analyzed, but this experimental model did not reflect the clinical postoperative condition. Therefore, we investigated the bone-resorbing cytokines interleukin and tumor necrosis factor (IL-1α, IL-6, TNFα) produced by continuous PE administration using histomorphologic findings and the reverse transcription polymerase chain reaction. TNFα mRNA was continuously detected in the PE continuous-infusion group, whereas it was not expressed in the PE single-injection group. IL-1α and IL-6 mRNA were detected in both groups. As for histomorphological findings, in the PE continuous-infusion group proliferating fibrous tissue penetrated subchondral bone and the interface membrane around the K-wire inserted into the femur. Numerous osteoclasts were observed in the vicinity of resorbed bone. In the PE single-injection group, proliferation of fibrous tissue with bone resorption was not observed. TNFα may play a major role in fibrous tissue formation and osteoclastic bone resorption caused by PE debris. This osteolysis model is useful for investigating osteolysis associated with bone-resorbing cytokines under conditions similar to those seen with a human prosthesis.

Key words Rat model · Polyethylene particles · Bone-resorbing cytokines (IL-1α, IL-6, TNFα)

Introduction

Periprosthetic bone loss has been recognized as a type of osteolysis associated with failure of total joint arthroplasty.[25] Osteolysis around implants is reported to be caused by osteoclastic bone resorption stimulated by bone-resorbing cytokines released from macrophages phagocytosing wear debris, such as polyethylene (PE) particles.[7,16]

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Several in vivo studies have investigated bone–implant interface tissue by periodic injections of massive amounts of PE particles into the animal joint.[5,14,29] According to their histomorphological findings, fibrous tissues formed by single injections of PE particles did not produce active neocortical perforation and resorption. Few in vivo studies have investigated the relation between osteolysis and the presence of osteoclasts in fibrous tissue.[29] Moreover, few in vitro studies have analyzed bone-resorbing cytokines produced from macrophages stimulated by PE particles,[27,28] and no in vivo studies have analyzed gene expression of these cytokines in animal models to study the response to PE particles.

After joint replacement surgery, PE particles are generated in the joint continuously rather than periodically by daily joint motion. In our previous study, we developed a rat osteolysis model continuously infused with PE particles for a short period of time.[20] In this study, we studied an osteolysis model in the rat knee continuously infused with PE particles for a long period of time (10 weeks) using an osmotic pump. We analyzed the histomorphological findings and the patterns of gene expression of bone-resorbing cytokines in the fibrous tissue formed by continuous stimulation of PE particles and compared these findings to those obtained with single injections of PE particles.

Materials and methods

Materials

High-density PE particles remaining from a previous study and provided by 3M Health Care Limited (Tokyo, Japan) were used.[18] Particle size distribution was determined by the Coulter counter method (mean diameter 2 μm, particle weight 7.51×10^{-9} mg), and the particle shape was spherical. PE particles were sterilized by γ-rays (25 KGy).

For continuous infusion of PE particles, an osmotic pump (ALZA, Palo Alto, CA, USA) filled with 200 μl of sterile rat serum containing 0.1 mg of PE particles (1.33×10^7 particles/0.1 mg) was implanted subcutaneously in the back of rats (pumping rate 0.5 μl/h). The number of PE particles was 6.7×10^6 particles per week.

Operative techniques

A total of 116 female Wister rats (10 weeks old) were used in this study. The study protocol was approved by the Institutional Animal Care and Research Advisory Committee. (Tokyo Women's Medical College guidelines for the care and use of laboratory animals have been observed.) Each rat was anesthetized with intraperitoneal pentobarbital sodium. In the continuous-infusion group (40 rats), a knee joint was surgically exposed using a transpatellar tendon approach, and a 1.5 mm diameter drill hole was made using a sterilized stainless-steel wire (K-wire) in the intramedullary cavity from the femoral condyle. Then, another K-wire (1.0 mm diameter and 1.0 cm in length) was placed in the intramedullary cavity of the unilateral femur. An osmotic pump was implanted subcutaneously into the dorsum of the rats. Then, a sterilized polyethylene tube (outside diameter 1.22 mm, inside diameter 0.76 mm; Becton Dickinson, Sparks, MD, USA) connected to the osmotic pump was subcutaneously led to the knee and placed in the joint cavity. In the continuous serum-infused group (40 rats), the same the operation was done using a pump filled with only rat serum. Pumps were exchanged every 2 weeks. Eight rats from each group were killed postoperatively at 2, 4, 6, 8, and 10 weeks.

For single injections of PE particles, 18 rats were given injections every week of 100 μl of sterile rat serum containing 0.5 mg of 6.7×10^6 PE particles into the joint cavity of the unilateral knee with a 26-gauge hypodermic needle by a transpatellar approach. The number of PE particles in this study was similar to the number in the previous in vivo single-injection study, which was about 1.0×10^8 per 2 weeks.[5,9] In the single-injection serum group (18 rats), the same operation was done with only rat serum. Six rats were killed from each group postoperatively at 4, 8, and 10 weeks.

Tissue samples

For the histomorphological study, at each time point the femoral shafts of four rats from the PE continuous-infusion group, four rats from the serum continuous-infusion group, three rats from the PE single-injection group, and three rats from the serum single-injection group were suspended in 4% paraformaldehyde, 2.5% glutaraldehyde, and 0.067 M cacodylic acid buffer for 6 h, and then demineralized for 2 weeks in 4% ethylenediaminetetraacetic acid (EDTA) solution at 4°C. For the reverse transcription polymerase chain reaction (RT-PCR) study, we removed 100 mg of fibrous tissue around the drill hole in the knee joint of the remaining rats in each group at each time point.

Hematoxylin and eosin staining

After demineralization, the specimens were dehydrated using ethanol, embedded in paraffin after removing the ethanol using chloroform, cut into 4-μm sections, and stained using hematoxylin and eosin (H&E).

Enzyme histochemistry for tartrate-resistant acid phosphate

The same 4-μm sections were prepared for the tartrate-resistant acid phosphate (TRAP) test. TRAPase was detected simultaneously by Burstone's azo dye method.[6] For TRAPase staining, specimens were incubated in a mixture of 8 mg of naphthol bromohydroxynaphtholic acid-methoxyanilide phosphate, 70 mg of red violet 5-chloro-4-bezamido-2-methylbenzene diazonium chloride, hemi [zinc chloride] salt (Sigma, St. Louis, MO, USA), and 50 mM L(+)-tartaric acid (0.76 g) (Nacalai Tesque, Kyoto, Japan) diluted in 100 ml of 0.1 M sodium acetate buffer (pH 5.0). Incubation was performed for 30 min at 37°C, and the reaction field was counterstained with methyl green.

Analysis of gene expression by RT-PCR: RNA preparation and cDNA synthesis

Immediately after removing fibrous tissue in the knee joint, total RNA was isolated by the single-step guanidinium-thiocyanate-phenol-chloroform extraction procedure[8] using ISOGEN (Nippongene, Toyama, Japan). The quantity of RNA was estimated on an aliquot by spectrophotometry at 260 nm. After denaturation of freshly prepared RNA at 65°C for 10 min, single-strand cDNA was synthesized with random primers by reverse transcription (RT) at 37°C for 60 min in a 20-μl reaction mixture containing 1 μg of total cellular RNA; random hexamer 50 pmol/μl; RT buffer (50 mM Tris-HCl pH 8.3, 3 mM $MgCl_2$, 75 mM KCl); 0.01 M DTT; 1 mM dATP, dCTP, dGTP, and dTTP; 20 U of RNase inhibitor (Takara, Kyoto, Japan); and 100 U of MuMLV reverse transcriptase (Takara). After heating at 99°C for 10 min for denaturation, the cDNA was cooled on ice and used for amplification.

PCR amplification and detection of products

Enzymatic amplification of specific cDNA sequences by PCR was performed using a DNA Thermal Cycler 2400