Effects of polyethylene and TiAlV wear particles on expression of RANK, RANKL and OPG mRNA

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Background **Wear debris has been associated with periprosthetic osteolysis and loosening of total joint arthroplasties. RANKL (receptor activator of NF-κB ligand), RANK (receptor activator of NF-κB) and OPG (osteoprotegerin) are three key molecules which regulate differentiation, survival, fusion, and activation of osteoclasts.**

Material and methods **We evaluated the effect of TiAlV and polyethylene particles on expression of RANK, RANKL and OPG mRNA. We used a human monocytic leukemic cell line (THP-1) in this in vitro study. THP-1 monocytes were differentiated into macrophage-like cells and exposed to polyethylene particles and prosthesis-derived TiAlV particles. The supernantant was used for measurement of TNFα protein and total RNA was extracted from the cells. Expression of the genes coding for OPG, RANKL and RANK was analysed at the mRNA level using a semiquantitative RT-PCR method.**

Results **Both polyethylene and TiAlV particles induced a significant release of TNFα after 6 h of exposure and a significant upregulation of RANK mRNA. OPG mRNA expression was transiently upregulated after exposure to polyethylene and TiAlV particles. These effects were dependent on particle dose. RANKL mRNA was not detectable in our THP-1 model. In contrast, LPS exhibited a different pattern of RANK/RANKL/OPG mRNA expression.**

Interpretation **Our findings provide evidence that both polyethylene and TiAlV particles induce upregulation of RANK expression in cells of the monocytic lineage, which may be important for periprosthetic osteoclastogenesis.**

Periprosthetic bone resorption leading to aseptic loosening is a common cause of clinical failure of total joint replacement (Harris 1995). In vitro, in vivo and tissue retrieval studies have revealed that wear particles from prosthetic material stimulate mainly macrophages to release mediators, which initiate a process leading to periprosthetic bone resorption (Goodman et al. 1998, Kadoya et al. 1998). Besides other causes of aseptic loosening, such as surgical quality and implant design, the biological effects of wear particles are probably most important. In previous studies, we have demonstrated in an in vitro THP-1 model (human monocytic leukemia cell line) that both TiAlV and polyethylene particles induce the release of TNFα, IL8 and IL1 from differentiated THP-1 cells (Rader et al. 1999b, 2002). One knockout study focused on the essential role of TNFα in particle-induced osteolysis, as mice failing to express the p55 TNF receptor are protected from the profound osteoclastic bone resorption after subperiostal implantation of polymethylmethacrylate (PMMA) particles (Merkel et al. 1999).

It has been shown that human macrophages associated with wear particles, isolated directly from periprosthetic tissues surrounding loosened implants, can differentiate into multinucleate cells displaying all the functional and cytochemical characteristics of osteoclasts (Itonaga et al. 2000). The underlying cellular mechanisms and the manner in which the humoral factors contribute to activation and differentiation of osteoclasts are controversial. A recent study has shown that TNFα is sufficient to induce human osteoclast differentia-

tion from arthroplasty macrophages independent from the RANK (receptor activator of NF-κB) signalling pathway (Sabokbar et al. 2003). On the other hand, RANKL (receptor activator of NF-κB ligand) knockout mice exhibit typical osteopetrosis with total occlusion of bone marrow space due to absence of osteoclasts (Kong et al. 1999). RANKL, RANK and OPG (osteoprotegerin) are three key molecules which regulate differentiation, survival, fusion, and activation of osteoclasts (Khosla, 2001). However, Lam et al. (2000) have shown that TNFα induction of osteoclastogenesis occurs as a result of direct stimulation of macrophages that are exposed to a stromal enviroment expressing permissive levels of RANKL. On this basis, they concluded that TNFα alone does not induce osteoclast formation.

A recent study has shown that titanium alloy (TiAlV), cobalt chrome-(CoCr) and 316L stainless steel (SS) particles have effects on expression of RANKL, RANK and OPG mRNA (Haynes et al. 2001). Polyethylene particles, which have the most important clinical relevance among all wear particles, were not examined. The biological effects of polyethylene particles on the involvement of the OPG/RANKL/RANK system has not been described. Characterization of pathogenic pathways of aseptic loosening in relation to particle composition is a prerequisite for the development of new prophylactic and therapeutic approaches in the future. In this in vitro THP-1 study, we evaluated whether polyethylene and TiAlV particles induce upregulation of RANK expression in cells of the monocytic lineage.

Material and methods

Particles

Titanium (Ti) alloy particles were isolated from soft tissue membranes harvested at revision surgery (Maloney et al. 1995). The patient suffered from heavy metallosis after complete wear of polyethylene inlay, leading to contact of the tibial-component (CrCoMo) with the femoral-component (TiAlV). This resulted in accumulation of great numbers of TiAlV particles. The implant was a Microloc prosthesis with a metal-backed patella. The implant duration was 10 years. The tissue was digested by treatment with concentrated nitric acid, followed by a centrifugation step for 30 min at 10 000 rpm. The wear particles were washed three times with PBS. Then trypsin was added in order to digest the remaining proteins. The mixture was incubated for two hours at 37 °C and consisted of: 200 mg wear particles, 100 mg trypsin, 100 mg CaCl2, 100 mL PBS at pH 7.4. Afterwards, the particles were washed three times with PBS and recovered. Before incubation with cells, the metal particles were heated to 180 °C for 3 h to avoid bacterial and endotoxin contamination.

The polyethylene particles (UHMWPE) were similar to those used in our previous studies (Rader et al. 1999a, b) and were obtained from Johnson & Johnson (Norderstedt, Germany).

Analysis of particles

The size of particles was determined with a particle size analyzer (Coulter Electronics, Luton, United Kingdom) and by scanning electron microscopy (DSM 940, Zeiss, Oberhocheln). An edx-analysis (energy dispersive X-ray-analysis, QX2000, Link Analytical, Oxford, GB) was performed. The diameter of particles was 0.1 (SD 0.2) μm for TiAlV and 1.13 (SD 1.91) μm for polyethylene (data not shown). TiAlV particles were recycled by digesting and sterilizing again as described above. Polyethylene particles were discarded after each cell exposure. The particle preparations and media we used contained less than 10 pg/mL endotoxin (E-toxate kit, Sigma Chemical Company, St. Louis, MO). When 10 pg/mL endotoxin was added to the macrophage cultures, the production of TNFα was not affected, indicating that endotoxin contamination was not responsible for any of the effects seen in this study (data not shown).

Cell culture

THP-1 cells (ATCC, Rockville, USA) were maintained in 250-mL culture flasks (Falcon) using RPMI 1640 medium supplemented with 10% fetal calf serum and 1% L-glutamine in a 5% CO_2 environment at 37 °C (Tsuchiya et al. 1980, Auwerx 1991). To improve phagocytotic activity, THP-1 cells were differentiated into macrophage-like-cells using 1,25-dihydroxy cholecalciferol (Sigma) at a final concentration of 10^{-8} mol/L. Thereafter, 10^{-7} mol/L GM-CSF (Duphar, the Netherthelands)

was added to the cells for 2 days, to promote further differentiation.

The macrophage-like-cells were exposed to wear particles in RPMI 1640. For a dose-response analysis, they were exposed at three concentrations (1×10^7, 1×10^8, 1×10^9 particles/mL). A time course was performed at 6h, 24 h, and 48 h in an enviroment of 5% CO2 at 37 °C. At each time point, a positive control with a final concentration of 1 μg/mL LPS (Escherichia coli 055:B5, Sigma-Aldrich, MFCD00283703) and a negative control that was neither treated with LPS nor with particles was performed. At each time point, total RNA was isolated from the pellet and the supernantant was used for measurement of TNFα. A rotation device described in previous studies was used to achieve good contact between cells and the suspended polyethylene particles (Rader et al. 1999b).

TNFα-assay

Levels of TNFα were measured in duplicate by a commercially available enzyme-linked immunosorbent assay (Milena, DPC Biermann GmbH, Bad Nauheim and Coulter Immunotec, Hamburg, Germany). Results presented represent the mean of three independent experiments (SEM).

Cell viability

Cell viability was assessed by Trypan blue staining. After incubation with particles or LPS, cell viability was no different to that of untreated samples at each time point and ranged between 95 and 99% (data not shown).

Total RNA extraction and DNase I digestion

Total RNA from cell pellets, synovial or periprosthetic membrane was extracted by the single-step guanidinium-isothiocyanate method using a commercial reagent (TRIZOL, GIBCO BRL, Life Technologies). DNase I digestion of RNA was performed as described (37). The concentration and integrity of extracted RNA were analysed by $OD_{260/280}$ measurement and gel electrophoresis.

RT-PCR

Equal amounts of DNase I digested RNA were used as templates for cDNA synthesis. RNA was reverse transcribed in duplicate by oligo-dT priming with Moloney murine leukemia virus reverse transcriptase (Superscript, GIBCO BRL, Life Technologies). After denaturation of freshly prepared RNA at 70 °C for 10 min, single-stranded cDNA was produced by reverse transcription at 42 °C for 45 min in a 20-μL reaction mixture containing 3 μg total RNA, 1 μL oligo-dT, 4 μL 5 × first-strand buffer, 2 μL 0.1 M DTT, 1 μL dNTP mix, and made up to 20 μL with DEPC-treated water. The amount of cDNA was quantified by OD260/280 measurement and gel electrophoresis.

2 μg of each cDNA sample was amplified by PCR in a total volume of 50 μL using 0.5 units of Ex Taq polymerase (Takara Shuzu, Otsu, Shiga, Japan) and specific oligonucleotide primers at 10 pmol/μL. In each RT-PCR reaction, endogenous 18S ribosomal RNA was reverse transcribed and co-amplified as a control to adjust for differences in cDNA input. PCR amplification was performed for 18S (ACCESSION X03205; sense primer: 5´-tcaagaacgaaagtcggaggttcg, position 1025-1048; antisense-primer: 5´-ttattgctcaatctcgggtggctg, position 1464-1487), OPG (ACCESSION U94332; sense primer: 5´-aattgcagtgtctttggtctc, position 587-605; antisense-primer: 5´-gccgctgcacgctgttt, position 928-944), RANKL (ACCESSION AF019047; sense primer: 5´-agcgtcgccctgttcttctattt, position 315-337; antisense primer: 5´-gccatccaccatcgctttctctg, position 553-575) and RANK (ACCESSION AF018253; sense primer: 5´-TTGCCCGGTTTAATAATTCTGCTTCTCTTC, position 669-698; antisense-primer: 5´-TGTATTCATCTTCTGTGGGCATCTGTCTG, position 1058-1086) on a Gene Amp 9600 Thermal Cycler (Perkin-Elmer Corp., Norwalk, CT). Amplifications were performed for between 18 and 35 cycles and each cycle consisted of denaturation at 94 °C for 30 s, annealing at 62 °C for 1 min, and extension at 72 °C for 1 min. Amplified products were separated by electrophoresis on a 1% agarose gel using a 100-bp DNA ladder (GIBCO BRL, Rockville, MD, USA) as a marker. Gels were stained with ethidium bromide (0.5 μg/mL) and the PCR products were photographed with a digital camera (Kodak 2.02). For each PCR sample, a negative control reaction without template was included, and to control for residual DNA contamination in the RNA preparations, for each RNA sample an RT reaction without reverse transcriptase was subjected to PCR (RT-minus, not shown). Only

RT-PCR reactions with negative results in the corresponding RT-minus samples were used for quantification. Band intensities were quantitated digitally using Scion Image software (Release Beta 3b, Scion Corporation, Maryland) and values were normalized to the intensity of the 18S bands. Data are expressed as means of three different independent RT-PCR reactions (SEM).

All PCR products were sequenced and found to be identical to the published sequences.

In each PCR procedure, the number of cycles was chosen to yield linear amplification kinetics.

Statistics

All values are expressed as means (SEM). Statistical analysis was performed using repeated measure ANOVA.

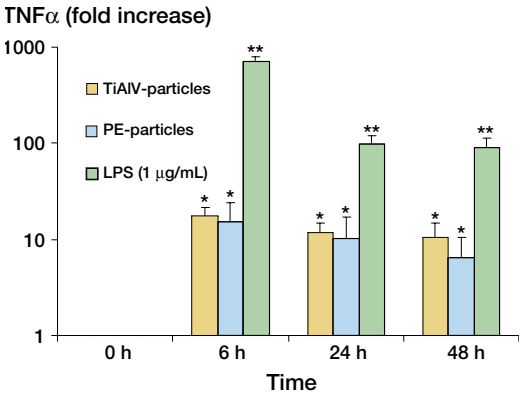

Figure 1. Time courses of TNFα concentrations in supernantant when differentiated THP-1 cells were exposed to wear particles or LPS. Values are expressed as fold increase in comparison to the control at the same time point. The ordinate is logarithmic. Data are expressed as means of three independent TNFα measurements (SEM). *($p < 0.05$), **($p < 0.01$).

Results

Wear particles induce secretion of TNFα from differentiated THP-1 monocytic cells

Consistent with our previous studies (Rader et al. 1999a, b), we observed that polyethylene and TiAlV particles induced a dose-dependent release of TNFα protein in differentiated THP-1 cells (Figure 1). When differentiated THP-1 cells were exposed to particles for 6 h the TNFα-concentration in the supernantant was 17 fold for TiAlV particles and 15 fold for polyethylene particles compared to the controls ($p = 0.02$). LPS, which is known to be a potent TNFα stimulus and a strong inducer of bone resorption (Chiang et al. 1999), induced a response to almost 600 fold ($p = 0.003$). Maximum levels of TNFα were reached after 6 h of incubation with LPS or wear particles. Upon LPS stimulation, TNFα concentrations decreased at 24 h, but were higher than the concentrations in both particle groups at all time points (Figure 1). The TNFα responses induced by particles were dose-dependent in differentiated THP-1 cells, as described in our previous studies (Rader et al. 1999a, b).

PE- and TiAlV-particles induce expression of RANK mRNA in differentiated THP-1 monocytic cells

In differentiated THP-1 cells, we observed a time-dependent upregulation of RANK mRNA expression up to 48 h after exposure to either TiAlV or polyethylene particles (Figure 2 A). The upregulation after exposure to polyethylene particles was earlier, so that we observed a significant increase after 24 h in comparison to the control (Figure 2 B). Relative to untreated samples, TiAlV particles induced up to a 3.5-fold increase ($p = 0.04$) and polyethylene particles a 3-fold increase ($p = 0.04$) after 48 h. LPS also induced a significant, but transient, upregulation of RANK mRNA expression after 6 h of incubation ($p = 0.05$), followed by a decrease (Figure 2 C). At later time points, i.e., 24h and 48h, there were no significant differences to initial expression levels in LPS-treated samples. The effects of polyethylene and TiAlV particles on RANK mRNA expression were dose-dependent (Figure 3). Wear particles induced higher maximal relative expression levels (up to 5-fold higher at 109 particles/mL) in comparison to LPS (2.5-fold higher expression). Thus, in comparison to LPS, wear particles induced a later but marked upregulation of RANK mRNA expression. LPS induced only a slight and transient upregulation of RANK mRNA expression.

Polyethylene and TiAlV particles induce transient upregulation of OPG mRNA expression in differentiated THP-1 monocytic cells

In contrast to RANK mRNA expression, time courses of OPG mRNA expression were simi-

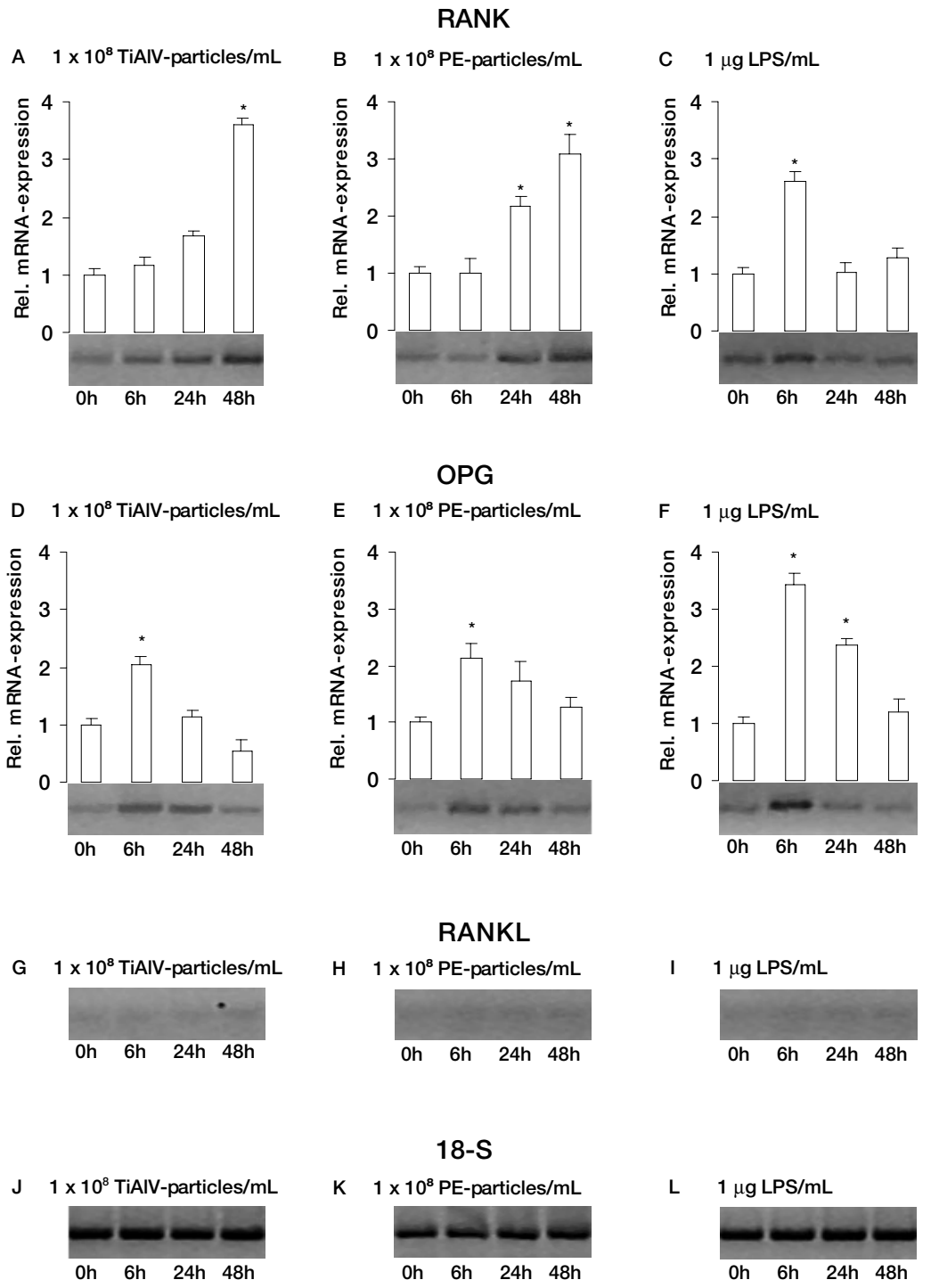

Figure 2 A-L. Time courses of mRNA expression of the constituents of the OPG/RANK/RANKL system in differentiated THP-1 cells exposed to particles and LPS. Values are normalized to 18S RNA and expressed as fold increase in comparison to control at the same time point. Data are expressed as means of three independent RT-PCR reactions (SEM) *($p < 0.05$). Representative gels are shown.

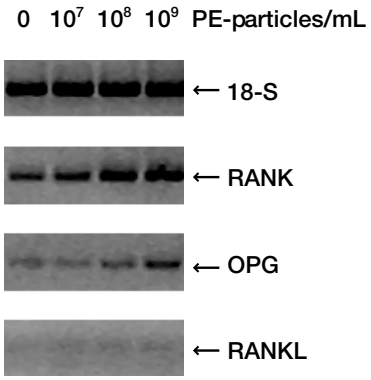

Figure 3. Dose-dependent expression of OPG/RANK/RANKL mRNA in activated THP-1 cells upon PE-particle exposure at different concentrations. We observed similar results after TiAlV particle exposure (data not shown). Representative gels are shown.

lar after exposure to LPS, or to either TiAlV or polyethylene particles (Figure 2 D-F). In both groups the maximum levels were seen after 6 h of exposure, followed by a successive decrease. In comparison to initial levels, the maximum levels were 3.3 fold in the LPS group and about 2 fold in the polyethylene and TiAlV particle group. The decrease in OPG mRNA levels after particle exposure was delayed in comparison to LPS. After 24 h, the expression of OPG mRNA was significantly elevated ($p = 0.05$) upon exposure to TiAlV or polyethylene particles, but not upon exposure to LPS. After 48 h in all groups, OPG mRNA expression levels were not significantly different to their initial levels. The effects of wear particles on OPG mRNA expression were dose-dependent (Figure 3), but the maximum levels of relative mRNA expression were lower for OPG (2.5 fold) than for RANK (≤ 5 fold). Thus, wear particles induced only a slight upregulation of RANK mRNA expression. In contrast, LPS induced a marked and prolonged upregulation of OPG mRNA expression. RANKL mRNA expression was not detectable in our in-vitro THP-1 model.

Discussion

Our data support recent findings that the OPG/RANKL/RANK system may be involved in wear particle-induced periprosthetic osteolysis. Our study extends and shows for the first time that it is not only TiAlV, SS or CrCo particles (Haynes et al. 2001), but also polyethylene particles, that can induce an osteoclastogenic pattern of OPG/RANKL/RANK mRNA expression. Numerous studies of wear debris from periprosthetic tissues have emphasized the most important role of polyethylene particles, and have demonstrated that 70–90% of the particles recovered are submicrometer polyethylene particles (Shanbhag et al. 1994, Campbell et al. 1995, Maloney et al. 1995, Hirakawa et al. 1996). Metal-on-polyethylene and ceramic-on-polyethylene components are the commonest bearing surfaces used in joint replacement, leading predominantly to generation of polyethylene particles.

Despite the great clinical relevance of polyethylene particles, most recent studies have examined other types of particle. This might be due to difficulties in sterilization and in avoiding endotoxin contamination. Moreover, polyethylene particles tend to adhere to each other and float on to top of the tissue culture medium, which makes them difficult to work with. We therefore used a rotation device, established in our previous study (Rader et al. 1999b), which has made comparative testing of metallic and polyethylene particles possible. The composition of the particles has been shown to affect cellular response. We chose to use differentiated THP-1 cells, in part because THP-1 cells have been used in previous immunological and biochemical studies to characterize monocytes and macrophages (Auwerx 1991). Also, THP-1 cells have been demonstrated to differentiate into mature macrophages after treatment with phorbol ester. In a rabbit model, Kubo et al. (1999) found that the material composition was more strongly correlated with histiocyte reaction than with particle size and total surface area. Furthermore, numerous studies have demonstrated differences in the pattern of cytokine release and toxicity between different particles. On the other hand, the different particles reported have similar effects, e.g., all particles induce the release of TNFα and IL1β in cells of the monocyte/macrophage lineage (Jacobs et al. 2001). This in-vitro study revealed similar effects of polyethylene and TiAlV particles on expression of OPG/RANKL/RANK mRNA. We observed significant upregulation of RANK

mRNA after treatment with both polyethylene and TiAlV particles. These findings complement the study by Itonaga et al. (2000) showing that arthroplasty macrophage-osteoclast differentiation occurs in the presence of soluble RANKL alone, and that this process is inhibited by OPG. On this basis, we suggest that TiAlV and polyethylene particles directly induce an upregulation of RANK on the surface of macrophages, so that these cells can receive the osteoclastogenic signal by RANKL.

As expected, expression of RANKL mRNA was not detectable in our in-vitro THP-1 model. To our knowledge, a relevant RANKL expression in THP-1 cells has not yet been described. RANKL mRNA is expressed at the highest levels in osteoblasts, stromal cells and lymphoid tissues (Kong et al. 1999, Khosla 2001). We determined RANKL in our in-vitro experiment for reasons of completeness. However, there is evidence that RANKL plays a major role in periprosthetic osteoclastogenesis; Haynes et al. (2001) detected RANKL expression in periprosthetic revision tissue. Furthermore, Hofbauer et al. (1999) revealed that TNFα and interleukin-1β, which are both abundant in periprostetic tissue surrounding loose implants, stimulate RANKL gene expression in human osteoblastic cells.

The third factor in the OPG/RANK/RANKL system is OPG, representing a secreted decoy receptor for RANKL. Both, in-vivo and in-vitro studies have demonstrated that OPG is an inhibitor of bone resorption (Yasuda et al. 1998). A mouse calvaria model has shown that ex vivo OPG gene therapy is effective in preventing wear debris-induced osteoclastogenesis (Goater et al. 2002). In our study, we observed only a transient and slight upregulation of OPG mRNA upon TiAlV and polyethylene exposure. These results suggest that there is a local overbalance of RANKL/RANK compared to OPG in periprosthetic tissue, leading to osteoclastogenesis. Furthermore, recent studies have shown that OPG not only binds to RANKL, but also to TRAIL (Suda et al. 1999). TRAIL is a tumor necrosis factor-related ligand which induces apoptosis upon binding to its different death domain-containing receptors. This suggests that OPG may have other functions besides inhibiting RANKL. A role in acute periprosthetic inflammation after wear particle contact can be postulated. In contrast, LPS, which is known to be one of the principal bacterial factors in stimulating bone resorption, induced a marked upregulation of mRNA for OPG, but only a slight and transient upregulation of RANK mRNA, suggesting that the pathways in the pathogenesis of osteolysis induced by LPS are not identical to that induced by wear particles. These findings are supported by another study (Chiang et al. 1999) showing that LPS-induced bone resorption is substantially mediated by TNFα and IL-1 receptor signaling. Moreover, the different time courses of expression of mRNA for OPG and RANK between groups indicate that the particle effects demonstrated are not due to LPS-contaminated particles or particle-associated endotoxin, as has been postulated by other authors (Bi et al. 2001, Brooks et al. 2002, Cho et al. 2002).

Our experiments demonstrate three important points relating to periprosthetic osteolysis. Firstly, RANK/RANKL/OPG is involved in periprosthetic osteoclastogenesis after both polyethylene and TiAlV particle exposure. Secondly, there is no significant difference in RANK/RANKL/OPG mRNA expression between exposure to polyethylene or TiAlV particles. In contrast, LPS exhibited a different pattern of RANK/RANKL/OPG mRNA expression. Thirdly, TNFα is markedly expressed after exposure to polythylene and TiAlV particles, and overwhelmingly expressed after exposure to LPS. Thus, direct induction of osteoclastogenesis by TNFα distinct from the RANK/RANKL signaling pathway, seems also to be important in wear particle and LPS-induced osteolysis (Azuma et al. 2000, Sabokbar et al. 2003). However, both pathways may act synergistically, mediated by a direct effect of TNFα on osteoclastogenesis and by an indirect effect of RANKL.

No competing interests declared.

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