The potential role of human osteoblasts for periprosthetic osteolysis following exposure to wear particles

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Abstract. Aseptic loosening in total hip replacement is mainly caused by wear particles inducing inflammation and osteolysis. Wear can be a consequence of micromotions at the interface between implant and bone cement. Due to complex cellular interactions, different mediators (e.g. cytokines, proteinases) are released, which can promote osteolytic processes in the periprosthetic tissue followed by loosening of the implant. Furthermore, a reduced matrix synthesis and an induced apoptosis rate can be observed. The purpose of this study was to evaluate to what extent human primary osteoblasts exposed to wear particles are involved in the osteolysis. The viability, the secretion of collagen and collagenases and the variety of released cytokines after particle exposure was examined. Therefore, human osteoblasts were incubated with particles experimentally generated in the interface between hip stems with rough and smooth surface finishings as well as different material compositions (Ti-6Al-7Nb, Co-28Cr-6Mo and 316L) and bone cement mantle made of Palacos R containing zirconium oxide particles. Commercially pure titanium particles, titanium oxide, polymethylmethacrylate and particulate zirconium oxide were used as references. The results revealed distinct effects on the cytokine release of human osteoblasts towards particulate debris. Thereby, human osteoblasts released increased levels of interleukine (IL)-6 and IL-8 after treatment with metallic wear particles. The expression of VEGF was slightly induced by all particle entities at lower concentrations. Apoptotic rates were enhanced for osteoblasts exposed to all the tested particles. Furthermore, the *de novo* synthesis of type 1 collagen was reduced and the expression of the matrix metalloproteinase (MMP)-1 was considerably increased. However, wear particles of Co-28Cr-6Mo stems seemed to be more aggressive, whereas particles derived from stainless steel stems caused less adverse cellular reaction. Among the reference particles, which caused less altered reactions in the metabolism of osteoblasts in general, ZrO_2 can be assumed as the material with the smallest cell biological effects.

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Introduction

A major problem in orthopaedic surgery is the aseptic loosening of endoprosthetic implants caused by abrasive wear particles (1). In particular at the interface between implant and surrounding bone cement abrasive wear particles can be released which lead to inflammatory reactions and the generation of mediators like cytokines and chemokines (2). The continuous generation and presence of particulate wear debris in the periprosthetic tissue results in a chronic inflammatory state, i.e. macrophages and foreign body giant cells release a variety of cytokines after phagocytosis of different wear particles (3-6). Hence, osteoblastic cells are suppressed, but osteoclastic cells show higher rates of proliferation leading to bone resorption. The consequence is weakening of the adjacent bone stock along with the aseptic loosening of the endoprosthetic implants and the necessity to revision surgery.

Particulate wear debris is known to activate a variety of cellular responses in macrophages, fibroblasts, osteoblasts and osteoclasts (7). These cell types are associated with the development of the periprosthetic osteolysis. Beside enhanced and chronic inflammatory reactions in the periprosthetic region, the cellular recruitment to this region is promoted by induced chemokine expression (8-12). For example, interleukine (IL)-8 and monocyte chemotactic protein (MCP)-1 are potent chemotactic agents that attract macrophages and monocytes. Both chemokines have been identified in the periprosthetic region associated with osteolysis (7). IL-6 affects long term human marrow cultures and forms multinucleated cells with many features of the osteoclast phenotype (13).

The vascular endothelial growth factor (VEGF) is a key regulator of physiological and pathological angiogenesis (14,15). For example, pathological angiogenesis is related to higher vascularisation of inflammatory areas and tumours providing their blood supply. Furthermore, VEGF is an indicator of several inflammatory conditions and also responsible for the migration of monocytes and the proliferation of several types of fibroblasts (16-18).

While cytokines may affect the bone metabolism negatively by the suppression of osteoblastic cells, collagenases directly destroy bone matrix by degrading different types of collagen. Vidovszky *et al* (19) compared tissue from osteolytic and non-osteolytic regions of failed cementless total hip replacements and found that the amount of collagenase, gelatinase, and stromelysin [matrix metalloproteinase (MMP)-1, MMP-2 and MMP-11] was elevated in the interfacial membranes from osteolytic regions.

However, it is not only an increased osteoclastic bone resorption due to particle exposure that can disrupt the balance in the bone remodelling process, but also a reduced bone formation caused by a direct negative impact of metallic particles on osteoblasts (20). As many authors already published, the synthesis of collagen type 1 as the major component of bone matrix can be considerably decreased (21,22).

The presented *in vitro* study is focused on the cellular effects of wear particles derived from the interface between hip stems with rough and smooth surface finishing as well as different material compositions and the surrounding bone cement. While the majority of published studies employed commercial particles and monocytic or osteosarcoma cell lines in *in vitro* experiments (2,11,23-25), the objective of this study was to evaluate generated wear particles of different implant materials regarding their effect on human, primary osteoblasts. We focused on particles derived from cemented titanium, cobalt chromium and stainless steel stems. After exposure with these wear particles the secretion of different cytokines (IL-6, IL-8, MCP-1 and VEGF) as well as the release of procollagen type 1 and collagen degenerating proteinase MMP-1 from the primary osteoblasts were analysed. Moreover, the influence of the wear particles on the programmed cell death of bone forming cells was examined and compared to commercially available particles. In this context, besides the already known role of osteoblasts for bone formation and integration of implants into bone, their relevance for destruction of bone by influencing the expression of proteinases or osteoclastogenesis will be estimated.

Materials and methods

Generation and preparation of abrasive wear particles. Particles were generated by simulating the interfacial wear between total hip stems and bone cement. This was achieved by using a special test apparatus (26). For particle generation cemented standard total hip stems made of either a Titanium alloy (Ti-6Al-7Nb), a Cobalt-Chromium alloy (Co-28Cr-6Mo) or stainless steel (316L) were employed. All stems were geometrically identical and characterised by a defined surface roughness (Rz value). A smooth surface finishing with a Rz value of approximately 7 μm, and a rough surface finishing with 20 μm were used. The cement mantle was manufactured from commercially available polymethylmethacrylate (PMMA) bone cement (Palacos® R, Heraeus Kulzer, Wehrheim, Germany), containing 15% zirconium oxide (ZrO_2) particles as radio-opaque additive.

The collected wear debris, i.e. a conglomerate of metallic and bone cement particles, was used for cell biological investigations. Thereby, the amount of Ti-6Al-7Nb, Co-28Cr-6Mo and bone cement particles was previously described by Lenz *et al* (27). Moreover, commercially pure titanium powder with an average particle size of 3 μm (cp-Ti; Grade E, dry; Chemetall, Frankfurt, Germany), pure polycrystalline ZrO_2 particles with mean size of 1.75 μm (Selectipur, Heraeus Kulzer) as well as particles out of PMMA (Heraeus Kulzer) and titanium oxide (TiO_2) with a mean size of 1.8 μm (BioCer GmbH, Bayreuth, Germany) were also used.

Five milligrams of each generated wear particles and commercially available reference particles were sterilized by the use of gamma radiation. Subsequently, a stock solution (10 mg/ml) was prepared by suspending the particles in 0.5 ml sterile PBS (PAA, Coelbe, Germany). Further dilutions with cell culture medium created the final particle concentrations of 0.1 and 0.01 mg/ml.

Isolation and cultivation of human primary osteoblasts. Human primary osteoblasts were isolated under sterile conditions from bone marrow derived from femoral heads of patients undergoing primary total hip replacement. All samples were collected after participants signed written informed consent forms and approval by the Local Ethics Committee (AZ: 2010-10).

Spongiosa specimens were extracted from the femoral heads, suspended in sterile phosphate-buffered saline (PBS) and washed twice. Osteoblasts were obtained from the resulting material by enzymatic digestion with collagenase A and dispase II (both from Roche, Prenzberg, Germany) in Dulbecco's modified Eagle's medium (DMEM, Biochrome AG, Berlin, Germany) supplemented with 1% penicillin/streptomycin as well as 1% amphotericin B (both from Gibco®-Invitrogen Cell Culture Products, Paisley, UK) for 3 h at 37˚C, 5% CO_2 and constant shaking. The resulting preparation was filtered through a cell strainer (70 μm pore size; BD Biosciences, Bedford, UK), centrifuged at 900 rpm for 10 min and the pellet was resuspended in complete medium. The complete medium was supplemented with 10% fetal calf serum (Gibco®-Invitrogen Cell Culture Products), 1% penicillin and streptomycin, 1% amphotericin B, 50 μg/ml L-ascorbate-2-phosphate, 10 mM β-glycerophosphate and 100 mM dexamethasone (Sigma-Aldrich, Munich, Germany).

Freshly isolated osteoblast-like cells were plated in 25 cm^2 culture flasks in 8 ml complete medium and incubated at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was changed every two days in the course of which non-adherent cells were aspirated. The progress of proliferation was determined by a microscopic control. After a confluency of 90% the cells were split in a 1:6 ratio. The cultivated cells were used in experiments at passage three. To verify the osteogenic character of isolated cells, alkaline phosphatase stainings were carried out.

For *in vitro* experiments, the osteoblasts were transferred to 96-well plates with 3,000 cells/well and 200 μl complete medium. The human osteoblasts were allowed to adhere for 24 h and then the particle-free medium was replaced by the respective particle-containing medium for 48 or 96 h, respectively. Osteoblasts incubated with normal culture medium served as test controls.

For the phenotypical characterisation of the bone cells the *de novo* synthesis of collagen type 1 was determined by means of ELISA tests. Furthermore, the effects on the apoptosis rate were investigated. Supernatants of osteoblasts incubated with particle-containing medium were investigated for the expression

of the cytokines IL-6, IL-8, MCP-1 and VEGF and the synthesis of the MMP-1. These data are intended to assess whether osteoblasts being bone forming cells contribute with their induction of osteoclast differentiation and matrix resorption to osteolysis after contact with abrasive wear particles.

Determination of the protein content. Total protein content in the monolayer was measured spectrophotometrically using the commercially available Roti®-Quant universal kit (Carl Roth, Karlsruhe, Germany) following manufacturer's instructions. Cells were treated with lysis buffer (1% v/v Tween, 100 mM PMSF), incubated for 10 min at room temperature and the lysates of 4-fold preparations combined in 1.5 ml reaction tubes. After centrifugation for 5 min at 13,000 rpm 50 μl of the supernatants were measured in triplicates in a 96-well plate. Each aliquot was mixed with 100 μl working solution (15 parts reagent 1 and one part reagent 2) and incubated for 30 min at 37˚C. Finally, light absorbance was measured at 490 nm on a spectrophotometer (Dynex Technology, Denkendorf, Germany). The total protein concentration was determined from a standard curve of absorbance vs. known protein concentrations [bovine serum albumin fraction V (Sigma-Aldrich)] run in parallel with experimental samples.

Determination of the apoptosis rate. The cleavage of cytoplasmic histone-associated DNA is characteristic for apoptotic cells. Therefore, the enrichment of mono- and oligonucleosomes in the cytoplasm of apoptotic cells was used as quantification of osteoblast apoptosis 48 h after exposure to the wear particles. In order to determine the histone-associated DNA fragments the commercially available Cell Death Detection ELISA^{PLUS} kit (Roche, Mannheim, Germany) was used following manufacturer's instructions. Before the analysis, cells were lysed for 30 min at room temperature. The cell lysates were centrifuged for 10 min at 200 x g and the supernatants were transferred into the streptavidin-coated microtiter plate for analysis. After adding 80 μl immunoreagent the assay was incubated for 2 h at room temperature. This was followed by rinsing each well three times with incubation buffer, pipetting of horseradish peroxidase substrate (ABTS) to the wells and incubating until colour development for 10-20 min. After adding 100 μl ABTS Stop Solution the light absorbance was measured at 405 nm (reference wave length 490 nm). The apoptosis rate was then normalised to the control treated group at each time point.

Characterisation of the de novo collagen type 1 synthesis. Type 1 collagen is the most abundant collagen in the bone, which is synthesised as a much larger procollagen precursor molecule and activated by enzymatic cleavage into the smaller collagen molecule. The sequence of the amino acids removed from the carboxyterminal end of the procollagen molecule is known as the carboxyterminal propeptide of procollagen type 1 (CICP). The determined level of procollagen 1 in the cell culture medium reflects the type 1 collagen *de novo* synthesis and is a useful marker for the metabolic activity of bone cells. The quantitative analysis of the carboxy-terminal propeptide (CICP) in culture supernatants was performed with a commercial immunoassay using monoclonal CICP-antibodies (Metra™ CICP EIA kit, Quidel, Buende, Germany). Medium was collected after 48 and 96 h of incubation and analysed following the manufacturer's instructions. Shortly, sample supernatant was diluted in a 1:12 ratio, filled in rabbit anti-CICP-coated wells of a 96-well plate and incubated for 45 min at room temperature. After rinsing each well three times with incubation buffer, a goat anti-rabbit alkaline phosphatase conjugate was added for another 45 min. This was followed by a final incubation period of 30 min with a working substrate solution and measurement of the light absorbance at 405 nm. The actual concentration of CICP is determined from a standard curve.

Expression of MMP-1. MMPs are zinc-depended endopeptidases with MMP-1 as a triple-helical collagen type 1 degrading enzyme. The quantitative analysis of the MMP-1 content in cell culture supernatants was performed with a commercially available enzyme-linked immunoassay according to the manufacturer's instructions (RayBio® human MMP-1 ELISA kit, RayBio, Norcross, USA). This assay employs an antibody specific for human MMP-1 coated on a 96-well plate. Standards and samples were pipetted into the wells and MMP-1 present in a sample was bound to the wells by the immobilised antibody. The wells were washed and biotinylated anti-human MMP-1 antibody was added. Subsequently, the unbound biotinylated antibody was washed away and HRP-conjugated streptavidin was added to the wells. The wells were again washed, a TMB substrate solution was added to the wells and colour was developed in proportion to the amount of bound MMP-1. The stop solution changed the colour from blue to yellow and the intensity of the colour was measured at 450 nm. The actual concentration of MMP-1 was determined from a standard curve.

Characterisation of inflammatory signals. To investigate the immunstimulatory effect of osteoblasts incubated with abrasive wear particles, the cytokines IL-6, IL-8, MCP-1 and VEGF were quantified in the cell culture supernatant after 48 and 96 h of incubation using the Multiplex Cytokine assay (Bio-Plex Pro Human Cytokines Group I 5-Plex; Bio-Rad, Munich, Germany). The assay is based on simultaneous multi-cytokine detection in a single well of a 96-well microplate involving fluorochrome-labeled microsphere beads. For the assay osteoblast supernatants, standard solutions and blanks are filled in the corresponding wells of a 96-well filter plate already containing anti-cytokine conjugated beads. After an incubation time of 1 h and several washing steps the secondary antibody was added for 30 min. Subsequently, streptavidin-phycoerythrin was added to the reaction mixture in order to bind to the biotinylated sites of the secondary antibodies. Finally, the beads were resuspended in assay buffer and detected by the Bio-Plex 200 System using the Bio-Plex Manager software 4.1.1 (both, Bio-Rad).

Cytokine concentrations were calculated using a standard curve derived from a recombinant cytokine standard supported by the Bio-Plex assay.

Flow cytometry. Flow cytometry was performed on primary human osteoblasts from two donors in passage three to exclude the presence of monocytes and macrophages in the cell culture. All steps of the following procedure were performed on ice. After harvesting the cells and preparing a single cell suspension (10^5 cells/500 μl PBS supplemented with

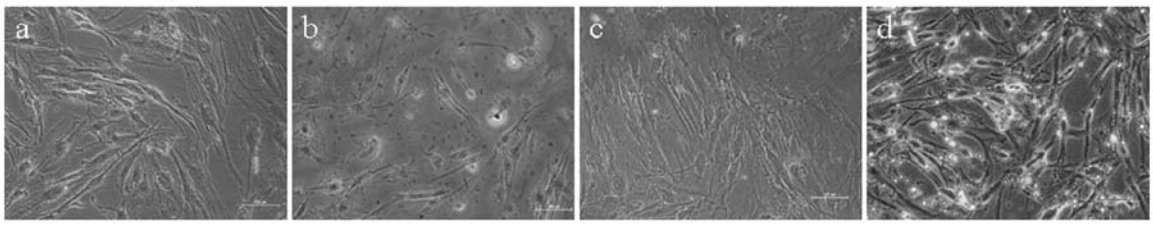

Figure 1. *In vitro* culture of human, primary osteoblastic cells after (a and b) 48 and (c and d) 96 h of incubation following treatment with 0.01 mg/ml particles from rough Ti-6Al-7Nb stem (b and d). Pictures were taken with a Nikon Eclipse TS100 microscope and a Nikon Digital Sight DS-2Mv camera.

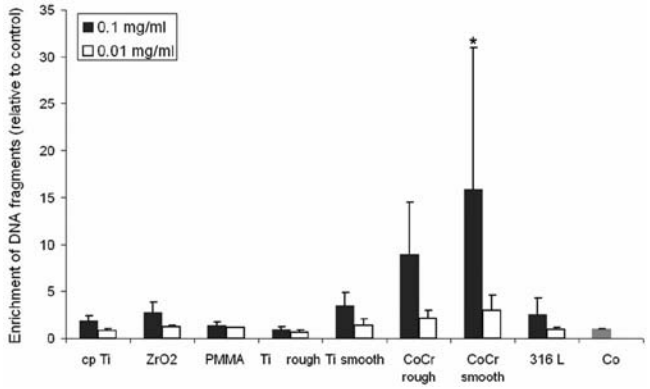

Figure 2. Quantification of osteoblast apoptosis 48 h after exposure to wear particles of different concentrations by analysing the enrichment of DNA fragments in the cytoplasm of osteoblasts compared to cp Ti, ZrO_2 and PMMA particles and a particle-free control (n=3). Values are means ± SD.

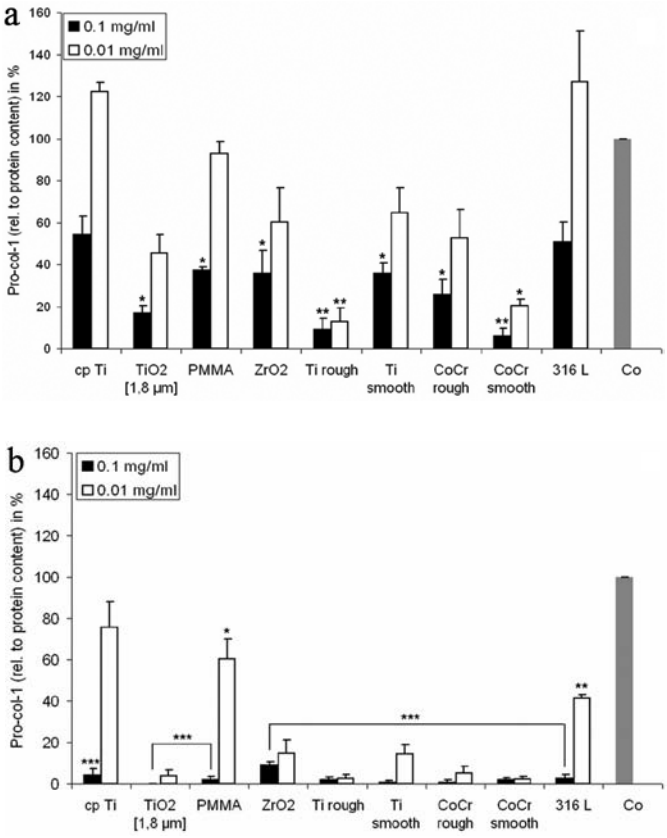

Figure 3. Changes in the procollagen 1 content in human osteoblasts (n=3) after exposure to wear particles of different concentrations after (a) 48 and (b) 96 h. The procollagen 1 amount is displayed relative to the protein content and related to the respective control (in %). Values are means ± SD.

1% bovine serum albumin [BSA (Sigma-Aldrich)], the cells were stained in the dark for 30 min with fluorescein isothiocyanate (FITC)-conjugated anti-CD68 (monoclonal antibody to human CD68, Acris Antibodies GmbH), phycoerythrin (PE)-conjugated anti-CD14 (monoclonal antibody to human CD14, eBioscience, San Diego, USA) and with mouse isotype (negative control, mouse IgG1-negative control-FITC, Acris Antibodies GmbH). The cells were then washed three times with PBS supplemented with 1% BSA and cell pellets were resuspended in 400 μl PBS supplemented with 1% BSA. Data were collected immediately after antibody-staining on a FACS Calibur cytometer (Becton-Dickinson, Franklin Lakes, NJ). Data analysis was performed with the CellQuest software (Becton-Dickinson).

Statistical analysis. For graphical representation the mean values ± standard deviation were presented. For all analysis human osteoblast cultures from three independent donors were used. The data of the different cell culture experiments were compared and statistical significances between groups were calculated with one-way ANOVA (post hoc LSD) using SPSS 15.0 for Windows (SPSS, Inc., Chicago). Significance was based on the untreated control at 48 h ($^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$).

Results

Cell viability. All different particle types affected the cell viability of human osteoblasts in a dose-dependent manner. The mean optical densities (using the MTT assay) obtained in former studies (27) were comparable for all treatment groups. Slight decreases of cell viability occurred after incubation with abrasive wear particles at a lower concentration (0.01 mg/ml) but a distinct decrease after the incubation with higher concentrated particle suspensions (0.1 and 1.0 mg/ml) was observed. Analysing the enrichment of mono- and oligonucleosomes in the cytoplasm of osteoblasts was used as quantification of osteoblast apoptosis 48 h after exposure to the wear particles. Though the amount of DNA fragments in osteoblasts incubated with lower particle concentrations (0.01 mg/ml) remained almost constant, more DNA fragments were detected in osteoblasts incubated with higher particle concentrations (0.1 mg/ml) compared to the control group (Fig. 2). An exceptionally negative effect of CoCr containing particles on osteoblasts was already found at lower concentrations. In contrast, particles from Ti-6Al-7Nb stems as well as commercial cp-Ti and PMMA applied in higher

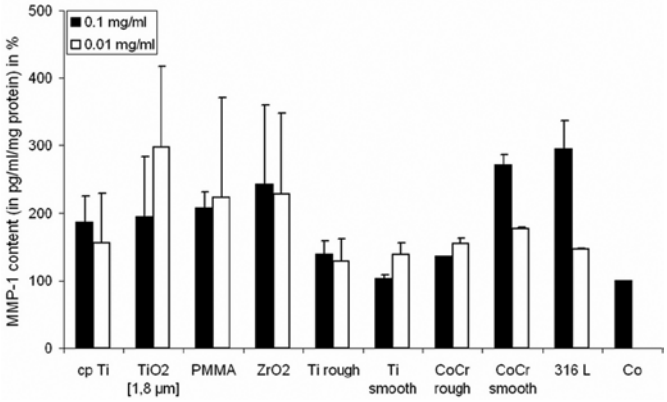

Figure 4. Changes in the MMP-1 content in human osteoblasts (n=2) after exposure to wear particles of different concentrations after 48 h. The MMP-1 amount is displayed relative to the protein content and related to the 48 h control (in %). Values are means ± SD.

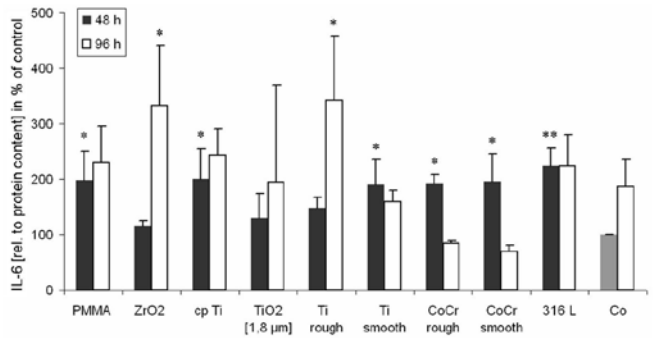

Figure 5. Changes in the IL-6 content in human osteoblasts (n=3) after exposure to wear particles (0.1 mg/ml) for 48 or 96 h. The IL-6 amount is displayed relative to the protein content and related to the 48 h control (in %). Values are means ± SD.

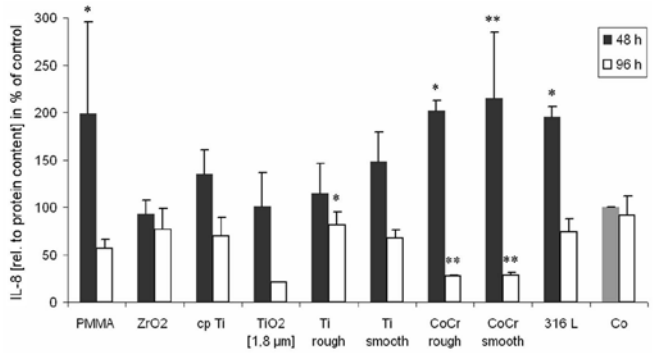

Figure 6. Changes in the IL-8 content in human osteoblasts (n=3) after exposure to wear particles (0.1 mg/ml) for 48 or 96 h. The IL-8 amount is displayed relative to the protein content and related to the 48 h control (in %). Values are means ± SD.

concentrations did not lead to an increase of DNA fragments. This reveals that the decreased cell viability analysed with the MTT assay results from the apoptosis of the affected osteoblasts whereas necrosis could not be detected.

Synthesis of collagen type 1. The *de novo* synthesis of collagen type 1 in osteoblasts was considerably inhibited after particle treatment. Metallic particles in the lower concentration (0.01 mg/ml) negatively affect the procollagen synthesis. This decrease was measured after 48 h of incubation and amplified after 96 h (Fig. 3). Only particles from the stainless steel stem and commercial PMMA powder at the lower concentrations had a negligible effect on the extracellular matrix protein production.

Higher particle concentration led to stronger inhibition of collagen synthesis. After 48 h (Fig. 3a) the amount of procollagen was decreased by 50% on average, after 96 h even more (Fig. 3b). Particles from rough titanium stems and cobalt chromium stems inhibited the release of procollagen type 1 stronger than the other particle types. After four days of particle incubation the production of the extracellular matrix protein collagen type 1 was nearly disconnected.

Matrix metalloproteinase 1 expression. MMP-1 is a zinc-dependent protease that degrades triple-helical type 1 collagen. After 48 h particle treatment the expression of MMP-1 in osteoblastic cells was elevated, which was already detectable at the lower concentration levels for all particle groups. Thereby, the highest increase of MMP-1 expression was found after exposure to ZrO_2, PMMA and TiO_2 reference particles (Fig. 4). Titanium containing stem materials only caused a minor increase of expression. Moreover, a higher particle concentration did not lead to a higher extension of MMP-1 generation (except smooth Co-28Cr-6Mo and 316L).

Interleukin-6. IL-6 is a marker for chronic inflammatory processes and is suspected to induce osteoclast generation. Osteoblasts cultured with different particle types released a higher amount of IL-6 compared to the non-stimulated control cells. This increase of IL-6 expression was already observed at the lowest particle concentration (0.01 mg/ml) after 48 h of incubation. In particular, particles from the Co-28Cr-6Mo stems and smooth Ti-6Al-7Nb stems increased the production of IL-6. Additionally, TiO_2 and cp Ti reference particles act as a stimulant for the IL-6 expression. The average expression was between 50 and 100% percent above the control group (data not shown) and increased further with higher particle concentrations (0.1 mg/ml) (Fig. 5). A longer incubation time (96 h) caused higher IL-6 levels in particle-treated as well as non-stimulated osteoblast cultures (Fig. 5). There was a tendency for cells incubated with either particles from hip stems or reference particles to release more IL-6 than untreated cells, but the differences in the expression levels were not as obvious as after 48 h of incubation. However, particles from the 316L and the Ti-6Al-7Nb stems had a major impact. In contrast, osteoblasts treated with particles from the Co-28Cr-6Mo stems in the higher concentration showed considerably decreased IL-6 levels after 96 h of incubation (Fig. 5). This may result from lower cell amounts caused by higher apoptotic rates in these samples.

Interleukin-8. Human primary osteoblasts express IL-8 which is among others responsible for osteoclast migration. After 48 h the IL-8 levels were only elevated in cultures treated with 0.01 mg/ml TiO_2 and PMMA reference particles as well as particles from the Co-28Cr-6Mo stems (data not shown). The

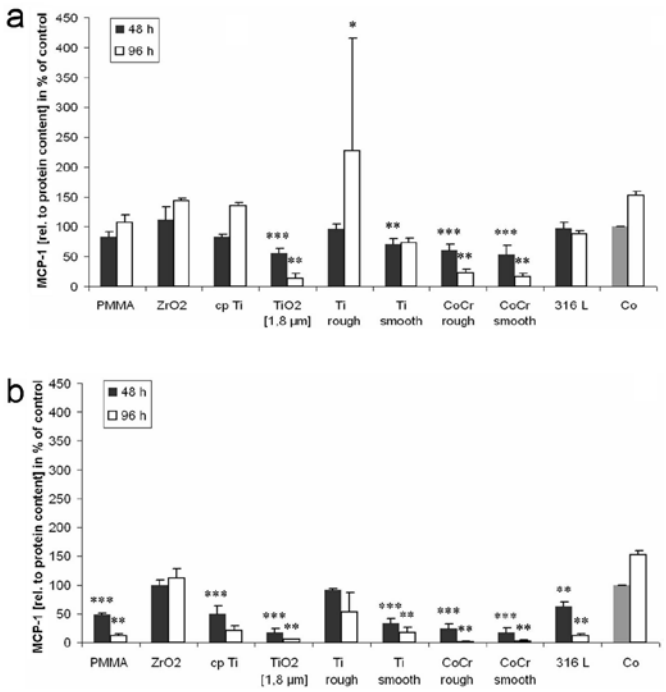

Figure 7. Changes in the MCP-1 content in human osteoblasts (n=3) after exposure to wear particles of different concentrations after 48 or 96 h. (a) 0.01 mg/ml, (b) 0.1 mg/ml. The MCP-1 amount is displayed relative to the protein content and related to the 48 h control (in %). Values are means ± SD.

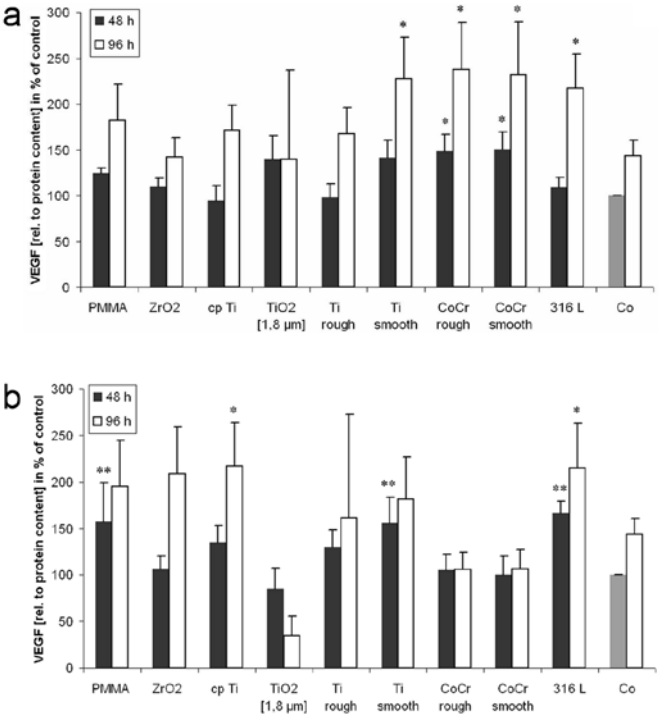

Figure 8. Changes in the VEGF content in human osteoblasts (n=3) after exposure to wear particles of different concentrations after 48 or 96 h. (a) 0.01 mg/ml, (b) 0.1 mg/ml. The VEGF amount is displayed relative to the protein content and related to the 48 h control (in %). Values are means ± SD.

other materials caused no variations above the control values at this concentration whereas higher concentrations (0.1 mg/ml) led to higher cytokine expression (Fig. 6).

The initial elevated expression of cytokine IL-8 inverted to a decreased expression after longer incubation. This is significant for TiO_2 reference particles as well as particles from both Co-28Cr-6Mo and rough Ti-6Al-7Nb stems. After 96 h in non-stimulated osteoblasts no differences of IL-8 levels were found compared to the earlier time point. However, the IL-8 levels were decreased for particle treatment at longer incubation periods, which was observed for all materials and was more pronounced after exposure to the higher particle concentration. Especially particles from the Co-28Cr-6Mo stems influenced the IL-8 secretion of osteoblasts (Fig. 6).

Monocyte chemotactic protein-1. MCP-1 as a further marker for osteoclast differentiation was slightly reduced after 48 h of incubation with different particle types at a concentration of 0.01 mg/ml (Fig. 7a). A higher particle concentration (0.1 mg/ml) caused a higher decrease in MCP-1 expression. Particles from the Co-28Cr-6Mo stems seemed to be more aggressive. TiO_2, cp Ti and PMMA reference particles also showed an inhibitory effect on the MCP-1 expression (Fig. 7b).

After 96 h the MCP-1 level in non-stimulated cultures was higher compared to the levels after 48 h. The reference materials at 0.01 mg/ml led to a reduction in MCP-1 expression relative to non-stimulated cultures although the amount of the cytokine was on average higher than after 48 h. Incubation with 0.1 mg/ml particles inhibited the secretion of MCP-1 considerably. The stem materials at 0.01 mg/ml led to a reduction of the MCP-1 expression relative to non-stimulated cultures, while the amount of the cytokine was not altered compared to the earlier time point (Fig. 7a). However, exceptions were the Co-28Cr-6Mo stem-derived particles. They caused a further decrease of the MCP-1 secretion at both concentration points.

Vascular endothelial growth factor. VEGF is a signal protein that can stimulate the growth of new blood vessels as well as the migration of monocytes or macrophages. After 48 h osteoblasts expressed VEGF. The release of this cytokine was considerably stimulated by treatment with the lower concentration (0.01 mg/ml) of both Co-28Cr-6Mo stems, the smooth Ti-6Al-7Nb and the 316L stem. After 96 h VEGF was further enriched in the particle and reference group (Fig. 8a). Equally to the above mentioned cytokines, particles from the Co-28Cr-6Mo stems at the higher concentration (0.1 mg/ml) (Fig. 8b) led to a more reduced VEGF expression.

Flow cytometry. Flow cytometry was performed representatively in samples of two donors. In primary cell cultures no CD68-positive cells (selective for monocytes) were detected, whereas CD14 (selective for monocytes and macrophages) was expressed in 2.43% and respectively 2.37% cells of the culture. These results confirmed that the findings can be ascribed to human primary osteoblasts (Fig. 9).

Discussion

There are many studies concerning the pathogenesis of aseptic implant loosening after total joint arthroplasty (28-30). Abrasive wear is presumed to play a key role for early implant loosening. In this context phagocytosis of wear particles leads

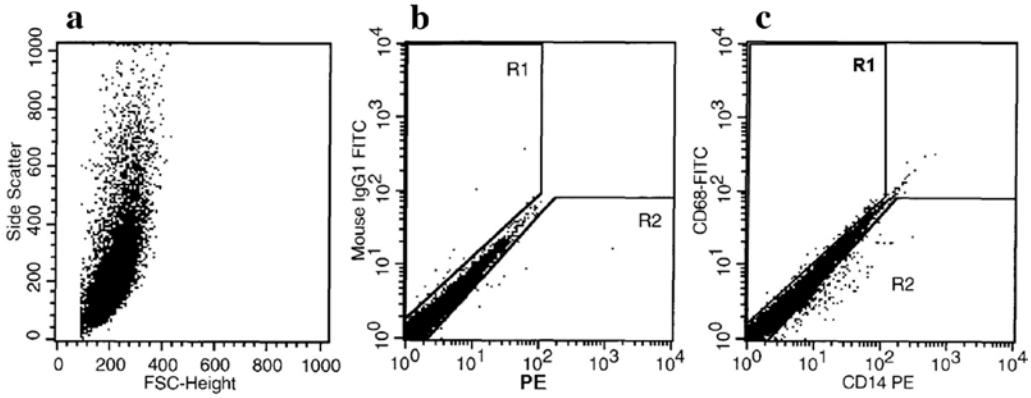

Figure 9. Fluorescence-activated cell sorting (FACS) of CD14 and CD68 expression in primary human osteoblast culture. (a) Side and forward scatter of unlabeled cells, (b) negative control and (c) representative example of primary human osteoblast culture.

to inflammatory reactions, osteolysis and tumour-like granulomatous tissue (28,31). The majority of *in vitro* cell studies have dealt with commercial particles and monocytic or osteosarcoma cell lines (2,11,23-25). In contrast, the present study focused on particles generated experimentally in a specific test apparatus simulating the micromotion between hip stem and cement mantle (26). Different abrasive wear particles were used for the investigations with human primary osteoblast-like cells.

Osteoblasts are extracellular matrix-producing cells and responsible for bone formation. Analyzing the synthesis of type 1 collagen, the degenerating enzyme MMP-1 and the apoptosis rate, the role of osteoblasts for matrix degeneration was examined. Furthermore, with the expression of the cytokines IL-6, IL-8, MCP-1 and VEGF the inflammatory impact of osteoblasts after incubation with wear particles was investigated. Moreover, differences between the abrasive wear of several stem materials and standard bone cement as well as reference particles were analysed.

When the synthesis of procollagen type 1 (CICP) is negatively affected, the *de novo* synthesis of type 1 collagen is considerably inhibited. Thereby, there are no significant differences between the various particle types according to Yao *et al* (21). In consequence, in the presence of abrasive wear particles the osteoblast-dependent matrix assembly is affected adversely and, therefore contributes to osteolysis. The second property of osteolysis is characterised by active matrix degradation. This process is activated by special proteinases (e.g. MMP-1) on the one hand and bone resorbing cell types on the other hand. Osteoclasts remove bone tissue and are formed by the fusion of cells of the monocyte-macrophage cell line (32). This differentiation can be induced by different factors, e.g. IL-6 stimulates osteoclast-like multinucleated cell formation from human marrow cells or macrophages (13). Together with MCP-1, IL-8 is chemotactic for lymphocytes, neutrophils and monocytes/macrophages at pico- and nanomolar concentrations. All three chemokines have been identified in the periprosthetic tissue associated with osteolysis (9,33-36).

Human primary osteoblasts release increased levels of IL-6 after treatment with metallic wear particles. This is most impressive after 48 h of particle incubation and is retained with increasing time. The enhancement was significant for both Co-28Cr-6Mo, the smooth Ti-6Al-7Nb and the 316L stems which is analogous at least for cobalt chromium to other findings (23,37). ZrO_2 particles did not influence the IL-6 expression. Therefore, the altered secretion of IL-6 seems not to be related to the particle type used as radio-opaque additive in bone cement. The lower expression of IL-6 after the exposure with higher particle concentrations may result from lower cell amounts caused by higher apoptotic rates. Especially particles from the Co-28Cr-6Mo stems compromised the viability of osteoblasts. Interestingly, there are no differences between the expression rates of the analysed cytokines and the surface finishing of the hip stems examined.

The expression of MCP-1 is considerably reduced after contact with wear particles, although osteoblasts are clearly able to express this cytokine. This is detectable after 48 and 96 h and significant for the Co-28Cr-6Mo, the smooth Ti-6Al-7Nb and the 316 L stems. Fritz *et al* (38) showed in bone marrow derived primary osteoblasts an early stress response with elevated levels of MCP-1 mRNA after 2 and 6 h and a decline after 12 h. Due to the more chronic character of abrasive wear, our experimental set-up focused on later time points. In the context of macrophage/monocyte activation it is well known that they become competent to perform certain functions in dependence of specific signals. MCP-1 not only attracts monocytes but may also cause cellular activation for specific functions in host defence (39). With respect to osteoblasts, MCP-1 seems not related to osteoclast differentiation by attraction of macrophages/monocytes. Maybe in combination with other cytokines (maybe IL-6 or IL-8) MCP-1 could be relevant for particle induced inflammation.

The IL-8 secretion is increased by osteoblasts after particle treatment. This was significant for Co-28Cr-6Mo stems and for the 316L as well as Ti-6Al-7Nb stems. Interestingly, the enhanced cytokine expression did not remain after longer incubation times. The decline of expression levels was also measured by other groups *in vivo* (40) and *in vitro* (38). Hence, by triggering IL-8 osteoblasts may attract osteoclasts and contribute to their migration into the periprosthetic tissue. However, the relatively higher expression levels of IL-8 compared to MCP-1 have also been detected by Fritz *et al* (38).

VEGF is an indicator of several inflammatory conditions and a key regulator of physiological and pathological angiogenesis (17). Our results show that osteoblasts express VEGF. The release of this cytokine was at least for the lower

concentration (0.01 mg/ml) significantly stimulated and after 96 h further enriched in the particle and reference group. By an increased content in the periprosthetic area the cytokine may attract monocytes and macrophages (17,41). Furthermore, within areas of elevated VEGF concentration enhanced blood vessel growth/angiogenesis can be observed. In the context of pathological angiogenesis the higher vascularisation of inflammatory areas and the vascularisation of tumours (42) for their supply may be mentioned. The secretion of VEGF from osteoblasts treated with different abrasive wear particles suggests that wear debris may induce tumourigenesis in the periprosthetic region. In summary, by expressing increased levels of IL-6, IL-8 and VEGF osteoblasts seem to participate in osteoclast differentiation. Remarkable differences between smooth and rough stems could not be assessed.

The relatively lower expression of IL-6, IL-8 and VEGF after the exposure with higher particle concentrations (primarily CoCr-derived particles) may result from lower cell amounts caused by higher apoptotic rates. In order to compensate the apoptotic rates of particle-treated cells and, therefore reduced cell amounts, all data were referred to the protein content of the cell cultures. However, osteblasts are matrix-secreting cells i.e. besides cell embedded proteins also extracellular matrix proteins were quantified, which results in measuring higher protein concentrations. Furthermore, the protein amount was quantified after 96 h of incubation but also used for quantification of the 48 h data. This could result in false higher protein content, which is not precisely proportional to the cell amount.

The results revealed distinct effects on the cytokine release of human osteoblasts towards particulate debris. The cytokine-induced differentiation of osteoclasts for matrix degradation could lead to enhanced degeneration of bone matrix. In addition, by the reduced synthesis of the extracellular matrix protein type 1 collagen, the regeneration of the bone matrix is further inhibited. Moreover, the increased expression of MMP-1 after particle contact would compromise matrix rebuilding and regeneration. The *in vitro* experiments of this study using particles reveal a release of MMP-1 in osteoblast cultures. Therefore, by the secretion of degradative enzymes, osteoblasts may actively contribute to matrix weakening.

Among the hip stem-derived particles Co-28Cr-6Mo induces the strongest cell reactions. This observation can also be obtained for the type 1 collagen synthesis and apoptotic rates in osteoblast cultures in comparison to other authors (21,25,43). The least aggressive stem-derived particle type seems to be 316L. Among the reference particles ZrO_2 showed slight reactions, although collagen synthesis (CICP) and degradation (MMP-1) were also affected. Thus, it can be assumed that mostly the content of metal particles in the wear debris is the most influencing variable in the culture experiments. All particle types tested, showed a similar size distribution (27) to those found in retrieval studies (44,45). For *in vitro* tests the size of the particles used should be ensured, since osteoblasts are capable of phagocytosis (44). For example, treatment of an osteoblast culture with particles from rough Ti-6Al-7Nb stems shows accumulation of the particles around the nucleus (Fig. 1).

The present study confirms that wear particles can alter the metabolism of human primary osteoblasts. In particular, metallic particles in the wear debris of cemented hip endoprostheses can compromise the vitality and activity of bone cells and bone matrix. In consequence, this may lead to a reduction of implant integration strength (46). Osteoblasts are rather responsible for bone formation, but can indirectly participate in bone degeneration by changing cell-viability and expression of specific chemokines as well as directly by the secretion of pre-osteolytic mediators and specific proteinases. Therefore, in total joint arthroplasty high releases of metallic particles should be avoided in order to reduce adverse cellular reaction and consecutive periprosthetic osteolysis.

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