Influence of metal ion solutions on rabbit osteoclast activities *in vitro*

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**Summary.** The purpose of the present study was to compare the effects of various metal ions (aluminium, chromium, cobalt, gold, iron, strontium, titanium and vanadium) on rabbit osteoclast activities, with respect to their number, size, resorptive capacity and their capacity to release proteinases. Marked heterogeneous osteoclastic behaviour was observed early in culture with metal ions (24 h) in term of resorption parameters. In contrast, protease activities (cysteine-proteinase and metalloproteinase activities) were not modulated in our culture conditions. Aluminium, iron, gold and titanium reduced the number of osteoclasts significantly. Aluminium and gold had no effect on osteoclast-mediated resorption on dentin-slices, although aluminium induced a greater number of very small lacunae. Titanium reduced only the mean surface area per lacunae, cobalt reduced the mean surface area of lacunae and increased their number, and iron reduced both parameters. Strontium had no effect on osteoclast formation and on total dentin slice surface resorbed. However, strontium increased the number of small lacunae formed on dentin-slices by osteoclasts. Chromium had no effect on osteoclast activities. These findings indicate that metal ions induce very early effects on osteoclasts, which can contribute to periprosthetic pathologies via different cellular mechanisms.

**Key words:** Osteoclast, Bone resorption, Metal ions, Prosthesis loosening

**Introduction**

Metal substrates (mainly, Ti, Cr, Co) and alloys are commonly used as biomaterials for the construction of total joint replacement devices. The functional activity of the cells is clearly influenced by the physicochemical properties of the implants with which they come in contact. For example, the type of substrate determines which integrins and extracellular matrix proteins are expressed by osteogenic cells (Gronowicz and McCarthy, 1996). Morphology and chemical composition, as well as the surface roughness of implants (Suzuki et al., 1997; Degasne et al., 1999), also appear to be essential factors. Osteoblast-like proliferation was found to be significantly higher on culture plastic than on both rough and smooth titanium disks, but greater on a rough than a smooth surface (Degasne et al., 1999). Similarly, exposure to metal wear particle preparations (titanium alloy, cobalt-chrome alloy) caused a dose-dependent reduction in the number of vitronectin-receptor positive osteoclasts formed (Neale et al., 2000).

The release of wear debris is considered to be the primary factor responsible for osteolysis-adjacent to implants. However, it is possible that metal ions released from the implant surface may also contribute to the loosening of these prosthesis. This suspicion arose in part from various retrieval studies of human hip arthroplasties, which have shown that in the surrounding tissues there are high concentrations of the implant metal components. Thus, for example, Betts et al. (1992) have measured the presence of metallic particles or ionic debris released by the components of total hip replacement in the surrounding tissues. The metal ions released from the implants may also contribute to prosthesis loosening by modulating bone cell activities (Aspenberg and Herbertsson, 1996; Gonzales et al., 1996). Thus, sublethal metal ion concentrations affect the formation and functions of bone cells (osteoblasts, osteoclasts) *in vitro* (Thompson and Puelo, 1995). Zinc ions modulate alkaline phosphatase activity (Hall et al., 2000) and inhibit osteoclast formation, but not osteoclast functions such as acid phosphatase and ß-glucuronidase activities (Yamaguchi and Kishi, 1996). Similarly, aluminium administration affected osteoblast and bone mineralization in the rat (Rodriguez et al., 1990). Oral fluoride and strontium supplementation in the mouse led to a rapid increase in the bone formation rate without detectable effect on osteoclastic bone resorption (Marie...
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Although metal implants have been used extensively as implant materials in different medical applications in recent decades, the factors and mechanisms underlying the biological response to metal ions release by wear debris are poorly understood, particularly in pathological situations. Moreover, most studies of metallic implants have dealt with the effects of metal wear particles on bone resorption mechanisms, and only a few have considered the influence of metal ions on osteoclasts. The purpose of the present study was to compare the effects of various metal ions (aluminium, chromium, cobalt, gold, iron, strontium, titanium and vanadium) on rabbit osteoclast activities, with respect to their number, size, resorptive capacity and their capacity to release proteinases.

Materials and methods

Reagents

Ion solutions of aluminium, chromium, cobalt, gold, iron, strontium, titanium, and vanadium were purchased from Sigma and were diluted in α-minimal essential medium (α-MEM) (Saint Quentin Fallavier, France). The pH of these ionic solutions was systematically adjusted to 7.4 until use. α-MEM, foetal bovine serum, antibiotic mixture and phosphate-buffered saline (PBS) were obtained from Gibco (Eragny, France). Twenty-four well tissue culture plates and plastics were supplied by Nunc (Roskild, Denmark), and glass coverslips from Polylabo (Strasbourg, France). May-Grünwald-Giemsia (MGG) for cytological staining and TRAP activity were revealed by the commercially available kit (N° 387-A) purchased from Sigma. Z-Phe-Arg-AMC substrate (7-N-benzoyloxy carbonyl-L-phenylalanyl-L-arginylamide-4-methylcoumarine), DTT (dithiothreitol), BCA (bicinchoninic acid), EDTA (ethylene diamine tetraacetic), E64 [L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane], proteic markers and bovine gelatin were obtained from Sigma. Recombinant GH (hGH Umatrope, 16IU) was a generous gift from the Lilly Company (Saint Cloud, France). Recombinant human IGF-I (hIGF-I) was supplied by R&D Systems (Abington, UK).

Total rabbit cell preparation and culture

Neonatal rabbit bone cells were isolated using a previously reported method (Rousselle et al., 2001). Briefly, 11-day-old rabbits were killed by cervical dislocation, and their long bones were isolated and freed from soft tissue, minced with scissors and placed in vortex in 20 mL α-MEM medium for 30 sec. After sedimentation for 2 min, the cell suspension was harvested (repeated twice). The cells were washed in α-MEM in a centrifuge (400 rpm for 3 min) and re-suspended in α-MEM supplemented with 10% foetal bovine serum and antibiotic mixture (100 U/mL penicillin, 100 µg/mL streptomycin). The cells were then seeded into 24-well plates at a density of 10⁷ cells/well on sperm whale dentin slices or on glass coverslips and the cultures were maintained in a humidified 95% air, 5% CO₂ atmosphere at 37 °C for 4 days.

Cathepsin activity measurements

Cathepsin K, B and L activities were determined by the action of a fluorogenic substrate, Z-Phe-Arg-AMC. Bone cells were cultured for four days, and each well was washed three times with α-MEM on the third day. Cells were then incubated for 24 h at 37 °C in serum-
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free α-MEM with or without ion solutions or 50 ng/ml hGH or 50 ng/ml hIGF-I. HGH and hIGF-I were used as positive controls (Rousselle et al., 2000). After this culture period, cathepsin activities were determined in supernatants, as described in Rousselle et al. (2000). In 96-well microtiter plates, 50 µl of cell culture supernatant were incubated in 140 µl of 100 mM sodium acetate buffer, pH 5.5, containing 1 mM EDTA and 0.1 mM freshly prepared DTT at 37 °C. The reaction was started by addition of 10 µl Z-Phe-Arg-AMC (5 µM assay concentration) and stopped after 4 h at 37 °C by addition of 100 µl of 100 mM sodium iodoacetate in 50 mM Tris/HCl buffer, pH 8.0. Assays were performed in triplicate with two control experiments: either a blank without a test sample, but containing all buffers and solutions, or addition of 10 µM E64 to the test sample before the substrate solution to inhibit any cysteine proteinase activities. In addition, a freshly prepared standard solution of 100 µl of free AMC in acetate buffer containing EDTA and DTT was used. For fluorescence, excitation and emission wavelengths were respectively 365 and 465 nm. The reaction rate was linear with time. In each well, adherent cells were lysed by 10 mM Tris, pH 7.4, 0.5% Triton X-100 and 0.05% sodium dodecylsulfate in 0.1 M freshly prepared DTT at 37 °C. Bovine serum albumin was used as standard. Specific cathepsin activities were then expressed as nmol of hydrolysed substrate.h⁻¹.mg protein⁻¹.

Measurement of matrix metalloproteinase activity

Matrix metalloproteinase activity was determined by zymography. Bone cells were cultured for four days, and each well was washed three times with α-MEM at the third day. Cells were then incubated for 24 h at 37 °C in serum-free α-MEM with or without ion solutions. After 24 h in serum-free medium culture, the viability of the cells was totally conserved. After this culture period, matrix metalloproteinase activities were detected in cell culture supernatants. Electrophoresis gel was composed of 10% polyacrylamide gel containing 0.1% gelatin. Gels were stained with 0.1% Coomassie brilliant blue (50% methanol, 7% acetic acid) and de-stained appropriately (20% methanol, 7% acetic acid, 3% glycerol). Proteinase activity was apparent as cleared (unstained) regions. The stained polyacrylamide gels were observed with a video camera, allowing transfer to software for image processing (Q500 Quantimeter, Leica, Cambridge, UK) and isolation of migration bands. Gelatinase activity was expressed as a percentage (shades of grey compared to the control). The stained polyacrylamide gels were then cut with a water-cooled diamond saw (Isomet), solutions, or addition of 10 µM E64 to the test sample before the substrate solution to inhibit any cysteine proteinase activities. In addition, a freshly prepared standard solution of 100 µl of free AMC in acetate buffer containing EDTA and DTT was used. For fluorescence, excitation and emission wavelengths were respectively 365 and 465 nm. The reaction rate was linear with time. In each well, adherent cells were lysed by 10 mM Tris, pH 7.4, 0.5% Triton X-100 and 0.05% sodium dodecylsulfate in 0.1 M freshly prepared DTT at 37 °C. Bovine serum albumin was used as standard. Specific cathepsin activities were then expressed as nmol of hydrolysed substrate.h⁻¹.mg protein⁻¹.

Assay for resorption pit formation by osteoclasts and determination of osteoclast size

The resorption activity of osteoclasts from the total rabbit bone cell preparations was estimated by pit formation on sperm whale dentin slices. Transverse sections of sperm whale dentin (about 100 µm thick) were cut with a water-cooled diamond saw (Isomet), stored in 70% ethanol/water and washed three times in culture medium before use. Cells were plated at a density of 10⁷ cells per well containing dentin slice for four days and each well was washed three times with α-MEM at the third day. Cells were then incubated for 24 h at 37 °C in serum-free α-MEM with or without ion solutions. After this culture period, the sperm whale dentin slices were sonicated for 2 min in distilled water to eliminate adherent cells and gold palladium-coated for SEM studies with a semiautomatic image analyser (Q500 Quantimeter). The surface of samples was then observed by scanning electron microscopy (JEOL 6300, Tokyo, Japan) operating at 15 kV to detect resorption pits. An imaging system (Q500 Quantimeter) was used to measure resorbed dentin surface. The resorption activity of cells was analyzed in terms of the percentage of dentin surface area resorbed, the number of lacunae and the mean surface of lacunae (µm²).

The effects of ion solutions on size of osteoclasts were determined by total rabbit bone cells cultured on glass coverslip in the conditions described above, washed three times in PBS and stained by cellular TRAP and May-Grünwald Giemsa staining. The number of TRAP-positive osteoclasts containing more than two nuclei and their size were determined under a light microscope (DMIRB, Leica) linked to a semiautomatic image analyser (Q500 Quantimeter).

Statistical analysis

All experiments were done three times in triplicate. The mean ± SD was calculated for all conditions and compared by ANOVA. Differences relative to a probability of two-tailed p<0.05 were considered significant.

Results

Titanium, aluminium, gold, iron and vanadium decrease the number of osteoclasts

The effect of metal ions on preformed osteoclasts was analyzed total rabbit bone cell culture in serum-free conditions. After 24 hours of culture, titanium down-regulated the number of osteoclasts in a dose-dependent manner. Thus, a maximal effect was observed at 10 µg/ml and at this concentration titanium reduced the number of osteoclasts about 2.2-fold (p<0.05) (Fig. 1a). Similarly, aluminium, iron and gold induced a significant decrease in the number of multinucleated cells in these culture conditions [about 1.6- (p<0.05), 1.5- (p<0.01), and 4.2-fold (p<0.01) respectively for 10 µg/ml metal ions] (Fig. 1a). Chromium, cobalt and strontium did not modify significantly the number of osteoclasts (Fig. 1b). In the presence of 10 µg/ml...
Vanadium, only very few osteoclasts can be detectable after 24 hours of culture (data not shown).

Modulation of osteoclastic resorption by metal ions without alteration of proteinase release

The influence of metal ions on the proteinase activities produced by bone cells was also studied. No modulation of cysteine-proteinase and metalloproteinase activities was observed in these culture conditions (data not shown), whereas hGH and hIGF-I, (used as positive controls) modulated MMP-2 and MMP-9 in similar conditions (Rousselle et al., 2000).

Aluminium, chromium, gold and strontium did not modify the total dentin surface area resorbed by osteoclasts (Fig. 2b). However, in the presence of 10 µg/ml aluminium or 10 µg/ml strontium, the number of

![Graph A](image)  
**Fig. 1.** Effect of metal ions on the number of osteoclasts from total rabbit bone cells. Total rabbit bone cells are cultured for 24 h on glass coverslips with or without metal ions (10 µg/ml). The number of osteoclasts is scored manually after tartrate resistant acid phosphatase staining. Results are the mean ± SD of three separate experiments performed in triplicate. *: p<0.05; **: p<0.01.

![Graph B](image)  
**Fig. 2.** Effects of metal ions on the resorption activity of osteoclasts from total rabbit bone cell culture. Total rabbit bone cells are cultured for 24 h on dentin slices with or without metal ions (10 µg/ml). Resorption parameters are analyzed by SEM coupled to a semiautomatic image analyser. a) number of lacunae per mm²; b) percentage of dentin slice surface area resorbed; c) mean surface of lacunae (mm²). Results are the mean ± SD of three separate experiments performed in triplicate. *: p<0.05; **: p<0.01; ***: p<0.001.
lacunae increased about 5.7-fold (p<0.01) and 3-fold (p<0.01) respectively as compared to the control without metal ion (Fig. 2a), whereas lacunal parameters (number and size) were not altered in the presence of these ions (Fig. 2a,c).

Iron and vanadium (10 µg/ml) reduced the dentin slice surface resorbed about 20-fold compared to the control without metal ions (p<0.01) (Fig. 2b). Similarly, the number of lacunae (Fig. 2a) and the mean surface area of lacunae (Fig. 2b) were significantly reduced in the presence of these ions.

In the presence of cobalt both parameters differed significantly from the control. 10 µg/ml cobalt up-modulated the number of lacunae per square millimeter by about 2-fold compared to the control without metal ions (p<0.05) (Fig. 2a), while the mean area of lacunae decreased about 2.5-fold (p<0.001) (Fig. 2c). These modifications did not result in a significant modulation of the percentage of dentin resorbed by osteoclasts (Fig. 2b).

Titanium, at a concentration of 10 µg/ml, produced no significant variation in the number of lacunae per square millimeter of dentin (Fig. 2a), but reduced the mean surface of lacunae significantly (Fig. 2c) and down-modulated the total surface area resorbed on the dentin slice (about 4-fold; p<0.05) (Fig. 2b).

**Effect of metal ions on osteoclast size**

Figure 3 shows the distribution of osteoclast size in the absence or presence of 10 µg/ml metal ions. Too few osteoclasts were observed in the presence of vanadium to allow analysis of size distribution because of the 90% decrease of viability in every cell type cultured (stromal cells and osteoclasts).

Aluminium, chromium, gold and strontium did not modulate osteoclast size significantly (data not shown), whereas cobalt and iron reduced osteoclast size (Fig. 3a,b). Thus, the median size of osteoclasts was 65.78 µm (n=279; mean ± SD: 72.5±56.2) and 71.16 µm (n=58; mean±SD: 76.9±14.9) in the presence of 10 µg/ml cobalt and iron respectively, compared to the control 86.67 µm (n= 250; mean±SD: 96.3±49.5) (p<0.001). Cobalt reduced the number of osteoclasts ranged from 90 to 130 µm of diameter (15.7% of cells as compared to 39% for the control) and up-modulated the number of smaller osteoclasts (65.4% from 60 to 90 µm compared to 36.9% for the control) (Fig. 3a). In the presence of iron, large osteoclasts (diameter>150 µm) disappeared totally, while the number of smaller osteoclasts increased.
Titanium up-modulated the size of osteoclasts present in the total rabbit bone cells in a dose-dependent manner (Fig. 4): 1 ng/ml had no apparent effect, whereas 100 ng/ml up-modulated the number of osteoclast ranged from 80 to 130 µm (68.6% of the cells as compared to 46.6% for the control). In the presence of 10 µg/ml of titanium, osteoclast size ranged from 70 to 100 µm, i.e., an increase of about 1.7-fold compared to the control (Fig. 4c). The median size of osteoclast was 95.01 µm (n= 242; mean ± SD: 107.6±31.04) (p<0.01) and 78.83 µm (n=246; mean ± SD: 88.5±31) in the presence of 100 ng/ml and 10 µg/ml titanium respectively, compared to the control 86.67 µm(n= 250; mean ± SD: 96.3±49.5) (p<0.01).

Discussion

It has been suggested that the osteolysis mechanism associated with prosthetic loosening involves wear debris resulting from alterations of prosthetic components and the release factors that activate bone resorption (Maloney and Smith, 1995). A net loss of bone at the tissue-implant interface could potentially occur from an increase in bone resorption by osteoclasts and/or from a decrease in bone formation by osteoblasts. Therefore, it can be hypothesised that metal ions may contribute to implant loosening by modulating the functions of osteoclasts or osteoblasts. Various cell culture systems derived from different species have been elaborated to study the molecular and cellular mechanisms of osteoclastogenesis and osteoclastic activities (Heymann et al., 1998). The model used in the present study (unfractioned neonatal rabbit bone cells) mimics some of the events in the periarticular region, mainly interactions between stromal cells, osteoclasts and their precursors (Roussel et al., 2000, 2001) thereby allowing investigation of the effects of many soluble factors such as ionic solutions on the activities of osteoclasts at early contact times.

It is estimated that up to 20% of total hip prostheses will show evidence of osteolysis within a decade of implantation (Jacobs et al., 1993). In addition to wear particles, metal ions released from the surface of implants may also play a role in the loosening of these prostheses, as suggested by the metal levels detected in tissue around metal implants (Dorr et al., 1990; Henning et al., 1992). Osteolysis results mainly from an inflammatory reaction when wear debris is phagocytosed by macrophages, which in turn release numerous proinflammatory cytokines (IL-6, IL-1β, TNF-α, etc.) (Rogers et al., 1997) involved in osteoclastic recruitment and differentiation from macrophages or mononuclear phagocytes (Heymann et al., 1998). Thus, wear particles cause an imbalance in the homeostatic equilibrium of cytokines and therefore of osteoblasts and osteoclasts. Moreover, the prostheses itself and the wear debris from its alteration release metal ions that may affect cellular metabolism. Elagi et al. (1995) found that titanium ions induced the up-modulation of acid phosphatase in peritoneal macrophages. In this context, Degasne et al. (1999) showed that commercially pure grade 3 titanium disks incubated in culture medium released titanium ions after as soon as 24 hours of incubation and in a time-dependent manner. Similar observations have been done in vivo and have revealed that titanium release can be detected near the implantation site of titanium fibres in the absence of systemic detection (serum, urine) (Bianco et al., 1996). Our data indicate that titanium affected all resorption parameters and that titanium ions may be involved in the control of prosthesis loosening. Titanium reduced the mean and total surface of lacunae, while the number of lacunae was not reduced. Therefore, titanium acted on osteoclastic resorption activity but did not modulate the number of mature osteoclasts. These observations are in agreement with those observed by Matsunaga et al. (2001) who demonstrated that addition of 10 µM titanium caused the degradation of osteoclast via the up-modulation of osteoclast apoptosis. These effects are probably similar to those resulting from the interference of the metal ions with the differentiation of bone marrow stromal cells into mature and functional osteoblasts (Thompson et al., 1995). However, Bi et al. (2001) demonstrated the reverse phenomenon. Thus, they revealed that titanium particles induced both murine cells and human peripheral blood monocytes to produce factors that stimulated osteoclast differentiation which led to a parallel increase in bone resorption. This discrepancy can be explained by the models used which did not conduct to osteoclast differentiation via the same differentiation pathway. The age of animals (adult mouse and 11-day-old rabbits) did not produce osteoclasts with a similar differentiation level (Roodman, 1996) and then the same capability to respond to various molecules. Moreover, in the experiments of Bi et al. (2001), titanium was used under particle form which in turn stimulates the phagocytosis mechanisms and then resorption. In the present study, titanium was used under ionic form which did not induce phagocytosis. These data, indicate clearly, that the effects of metals on bone resorption depend on the form used and on the cell differentiation level.

Though the percentage of dentin slice surface area resorbed was not affected in our study, aluminium acted on osteoclast survival and the size of lacunae. Aluminium reduced the number of osteoclast and their resorption capability (more small lacunae). Our findings also correlate with those of Lieberherr et al. (1997) who found that resorption activity was not modulated by high aluminium concentrations. If vanadium presented toxicity in our culture model, chromium had no effect on osteoclast number and activities. Iron affected all resorption parameters. Therefore, iron induced smaller osteoclasts from large osteoclasts as reported by Solari et al. (1995); or from the decrease in the cellular fusion process. In contrast, cobalt and strontium did not affect...
osteoclast number, only osteoclast activity. Thus, the total surface area resorbed was not a reliable indication of the effect of metal ions on osteoclasts.

The present study demonstrates that metal ions affect the capability of osteoclasts to resorb calcified matrix. Thus, iron, titanium, and vanadium reduced the number of osteoclasts and reduced the associated resorption via a decrease of the mean surface and the number of lacunae. The case of aluminium is different since it reduced the number of osteoclasts and did not reduce the dentin surface resorbed. This phenomenon is the result of an up-modulation of the number of lacunae with a lower mean surface area per lacunae. Such results have been already observed in presence of calcium phosphate ceramics (Yamada et al., 1997). Thus, the extent of osteoclastic resorption of calcium phosphate ceramic might, to a certain degree, be proportional to solubility of the material and then to the ion amounts released by the ceramic. Metal ions could act similarly on osteoclasts and could mimic physiological ions. The effects observed on osteoclasts following exposure to metal ions are still poorly defined. Two processes can be then hypothetized: the first concerns the interference of metal ions with osteoclast survival (apoptosis, formation of smaller osteoclasts) since less nucleated osteoclasts are able to form smaller resorption pits. The second concerns the effects of metal ions on the osteoclastic acidification pathway under the brush border. In this case, metal ions could interfere with the physiological ions (H⁺, Cl⁻, HCO₃⁻, Na⁺, K⁺) mainly implicated in the pH homeostasis of osteoclastic cytoplasm and acidification of the resorption chamber (Roussel and Heymann, 2002). In fact, both processes are probably involved.

Further studies are needed to define the cellular (cytological characteristics, etc.) and molecular (enzymatic activities, signal transduction, etc.) effects of metal ions released by implanted prosthesis on cells involved in bone resorption. Identification of the effects of metal ions is of paramount importance since it could contribute to the development of therapeutic strategies targeted to the prevention of aseptic loosening.

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