In vitro biomineralization by osteoblast-like cells
I. Retardation of tissue mineralization by metal salts

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Abstract

The cytocompatibility of stainless steel 316L (SS 316L) corrosion products was investigated with particular focus on the dose- and
time-effect of electrochemically dissolved SS and the corresponding separate metal ions on osteogenic bone marrow derived cells.
Type AISI 316L stainless steel (Fe 63.9%, Cr 18.0%, Ni 12.5%, Mo 2.8%, Si 1.2%, Mn 1.6% and C 0.025%, weight for weight) was
anodically dissolved in Hank's Balanced Salt Solution (HBSS) and diluted to the following concentrations: 500 μg mL−1 of Fe,
122 μg mL−1 of Cr and 101 μg mL−1 of Ni, as estimated by atomic absorption spectrometry. Similarly, salt solutions containing
500 μg mL−1 of Fe (FeCl3·6H2O), 122 μg mL−1 of Cr (CrCl3·6H2O) or 101 μg mL−1 of Ni (NiNO3) were prepared. All solutions were
diluted 1·102, 1·104 and 1·105 and their effects on cell proliferation and function of rabbit bone marrow cells were studied up to 28
days of culture. Bone marrow cells (second subculture) were cultured in α-Minimal Essential Medium (α-MEM) supplemented with
10% fetal bovine serum 10−8 mol l−1 dexamethasone, 2.52·10−4 mol l−1 ascorbic acid and 10−2 mol l−1 β-glycerophosphate. The osteoblast response to the presence of metal ions was evaluated by biochemical assays (enzymatic reduction of MTT for evaluation of cell viability/proliferation, and estimation of alkaline phosphatase (ALP) activity) and histochemical assays (identification of ALP positive cells and calcium and phosphates deposits). Results suggest a decrease in the expression of the osteoblast phenotype in the presence of ion and alloy solutions. Stainless steel corrosion products elicited slight effects but the corresponding metal ions produced
pronounced effects on the osteoblast phenotype, namely an alteration in the levels and temporal expression of ALP and lower and
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1. Introduction

The cellular events taking place at the bone/material interface can be determinant for the success of the implant long-term performance. Ion and particle release from metal implants remains a concern because of their potential role in various pathological bone and tissue conditions [1–5]. Knowledge of the toxic effects of ions on cells and tissues will be of significance in understanding the phenomenon of osteolysis and loosening of orthopaedic implants. Well-characterized osteoblast-like cell
cultures provide a suitable in vitro model to study the

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histochemical identification of calcium and phosphate deposits) found in control and treated cultures are reported.

2. Materials and methods

2.1. Metallic slurries

Type AISI 316L stainless steel (Fe 63.9%, Cr 18.0%, Ni 12.5%, Mo 2.8%, Si 1.2%, Mn 1.6% and C 0.025%, weight for weight) was anodically dissolved in Hank's Balanced Salt Solution (HBSS), which simulates the composition of physiological solutions. The resulting concentrations of the major metal ions in the 316L slurry were determined by atomic absorption spectrometry (AAS): 567 μg ml⁻¹ of Fe, 139 μg ml⁻¹ of Cr and 115 μg ml⁻¹ of Ni. Salt solutions containing 500 mg ml⁻¹ of Fe (FeCl₃·6H₂O), 122 μg ml⁻¹ of Cr (CrCl₃·6H₂O) or 101 μg ml⁻¹ of Ni (Ni(NO₃)₂) were prepared separately in HBSS.

2.2. Cell culture

Osteogenic cells were obtained from rabbit bone marrow. Briefly, one tibia was removed and washed twice with α-MEM (α-Minimum Essential Medium, SIGMA M 0894) containing antibiotic antimycotic solution (SIGMA A 9909) (1000 units of penicillin per ml, 10 mg ml⁻¹ of streptomycin and 25 μg ml⁻¹ of amphotericin B). Bone marrow pieces were distributed evenly onto a 14-cm diameter dish and covered with α-MEM supplemented with 10% fetal bovine serum, 10% antibiotic antimycotic solution, 10⁻⁸ mol⁻¹ dexamethasone, 2.52 × 10⁻⁴ mol⁻¹ ascorbic acid and 10⁻² mol⁻¹ β-glycerophosphate. A volume of 20 ml of supplemented medium was added 16 h later. Rabbit bone marrow cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air. Medium containing non-adherent cells was removed on day 4, and passages were done after tripasinization of adherent cells in exponential growth (7 days after the beginning of the incubation).

Cells of the second passage were grown for 7, 14, 21 and 28 days in control conditions and in the presence of stainless steel corrosion products and also the corresponding metal ions. Cells were seeded at a concentration of 10⁴ cells cm⁻²: (a) in 35-mm diameter tissue culture dishes for histochemical assays, (b) in 24-well culture plates for alkaline phosphatase activity assay and (c) in 96-well culture plates for cell viability MTT assay. Stainless steel corrosion products were added to some cell cultures in three concentrations, namely: SS⁻ (10⁻⁴ mol⁻¹ Fe + 10⁻⁴ mol⁻¹ Cr + 10⁻⁴ mol⁻¹ Ni). SS⁻ (10⁻⁴ mol⁻¹ Fe + 10⁻⁴ mol⁻¹ Cr + 10⁻⁴ mol⁻¹ Ni) and SS⁻ (10⁻⁴ mol⁻¹ Fe + 10⁻⁴ mol⁻¹ Cr + 10⁻⁴ mol⁻¹ Ni). Similarly, iron, chromium and nickel salt solutions were added separately to other culture dishes at equivalent concentrations, i.e. Fe⁻ (10⁻⁴ mol⁻¹ Fe + 10⁻⁴ mol⁻¹ Cr + 10⁻⁴ mol⁻¹ Ni), Fe⁻ (10⁻⁴ mol⁻¹ Fe + 10⁻⁴ mol⁻¹ Cr + 10⁻⁴ mol⁻¹ Ni) and Fe⁻ (10⁻⁴ mol⁻¹ Fe + 10⁻⁴ mol⁻¹ Cr + 10⁻⁴ mol⁻¹ Ni). For control experiments, normal culture medium was added to another set of cell cultures. The culture media were changed twice a week, collected and stored at −20°C for further analysis. Control and metal treated cultures were tested at days 7, 14, 21 and 28 to perform the biochemical and histochemical assays.

2.3. Biochemical assays

2.3.1. MTT assay

MTT assay (reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product) was used to estimate cell viability and proliferation. Cells (8 wells for each situation) were incubated with 0.5 mg ml⁻¹ of MTT in the last 4 h of the culture period tested. The media were then decanted, formazan salts were dissolved with 100 μl of dimethyl sulphoxide and the absorbance was determined at 600 nm in an ELISA reader.

2.3.2. Alkaline phosphatase activity

ALP was assayed based on the hydrolysis of p-nitrophenylphosphate (used as substrate) into p-nitrophenol at 37°C and pH = 10.3. After removal of culture medium, the tissue was washed twice with PBS (phosphate-buffered saline), detached from the culture dish with a rubber scraper after addition of PBS with Triton X-100 to each well, and sonified. Then, 100 μl of substrate (a 20 mmol⁻¹ solution) were added to 100 μl of each sample and the mixture was incubated at 37°C for 30 min. The reaction was stopped by the addition of 500 μl of NaOH 0.2 mol⁻¹. Optical density was determined at 405 nm on an ELISA reader and compared with the values of a series of p-nitrophenol standards.

Before performing the biochemical assays, it was observed that the metal ions present in the culture medium did not cause any interference in the MTT and ALP biochemical measurements.

2.4. Histochemical methods

The presence of ALP positive cells and phosphate or calcium deposits was visualized by histochemical staining. The cultures were fixed with 1.5% glutaraldehyde in 0.14 mol⁻¹ sodium cacodylate buffer and rinsed with distilled water.

2.4.1. ALP staining

Fixed cultures were incubated for 1 h in the dark with a mixture, prepared in Tris buffer pH = 10, containing
2 mg ml$^{-1}$ of Na-β-naphthyl phosphate (reacting substrate) and 2 mg ml$^{-1}$ of fast blue RR salt (diazotate-4-benzylamino-2,5-dimethoxyaniline, the diazonium salt that binds to phosphate ions to form a brown to black precipitate). The incubation was stopped by rinsing the samples with water and then the cells were observed in an Olympus BH-2 optical microscope. ALP positive cells stained brown.

2.4.2. Phosphate staining

Phosphate deposits were assessed by the von Kossa technique [10], i.e. the fixed cultures were covered with a 1% silver nitrate solution and kept for 1 h under UV light. After rinsing, a 5% sodium thiosulphate solution was added for 2 min and cultures were washed again. Phosphate deposits stained black.

2.4.3. Calcium staining

The fixed cultures were covered with a 1% S alizarin sodium sulphonate solution (0.028% in NH$_4$OH), pH = 6.4, for 2 min and then rinsed with water and acid ethanol (ethanol, 0.01% HCl) [10]. Calcium deposits stained red.

2.5. Statistical analysis

The percentage alternations of MTT reduction and ALP activity with respect to the values found in control and stainless steel treated cultures were calculated according to the formula:

\[
\% \text{ alteration} = \frac{[\text{value of absorbance of the tested metal ions} - \text{value of absorbance of the control}]}{\text{value of absorbance of the control}} \times 100.
\]

Data were analysed statistically using the double-sided t-test. All data presented represent the mean of at least three replicates (ALP) and the differences observed between groups were considered significant for $P$ values lower than 0.05.

3. Results and discussion

In the present work, rabbit bone marrow was cultured in experimental conditions reported to favour osteoblast differentiation in several bone cell culture systems, namely in the presence of ascorbic acid, β-glycerophosphate and dexamethasone [6–7, 11–12]. The second subculture was used to compare the effects of stainless steel corrosion products and the corresponding metal ions (Fe, Cr and Ni) on the long-term behaviour of osteoblastic cells.

In control conditions, cells proliferated gradually with incubation time, reaching a stationary phase around day 21; cultures presented high levels of ALP and maximal values were observed at approximately day 14; after that, activity of the enzyme dropped significantly [7]. Formation of mineral deposits, demonstrated by the positive staining of alizarin red and von Kossa assays, occurred in the third week of culture following the maximal ALP activity [7]. ALP has long been associated with biological calcification and these observations are in agreement with results reported in the literature for other culture systems [11, 12, 15–17, 19, 20, 22]

3.1. Cell viability/proliferation

Despite some controversy, reduction of the tetrazolium salt MTT by cellular enzymes has been widely used to measure cell viability/proliferation [22, 23] and to estimate drugs and biomaterials toxicity [6, 7, 23–28]. Osteoblast-like cells were cultivated in control medium or exposed to SS$^{-}$, SS$^{0}$ and SS$^{+}$ solutions, Fe$^{-}$, Fe$^{0}$ and Fe$^{+}$ solutions, Cr$^{-}$, Cr$^{0}$ and Cr$^{+}$ solutions or Ni$^{-}$, Ni$^{0}$ and Ni$^{+}$ solutions for 7 to 28 days. Fig. 1 shows results concerning cell viability/proliferation found in these cultures, expressed as percentage alteration relative to the control.

Stainless steel corrosion products used at different concentrations (SS$^{-}$, SS$^{0}$, SS$^{+}$) caused, in general, no significant effects ($P > 0.05$) in cell viability/proliferation, as compared to the control culture, although in the presence of SS$^{0}$ and SS$^{+}$ a trend for stimulation was observed, especially evident at day 21. In contrast, metal ions tested separately (Fe, Cr or Ni) at corresponding concentrations caused significant increase ($P < 0.05$) in cell viability/proliferation (Fig. 1b–d). However, a different pattern of variation was found in the presence of the three ions. In cultures exposed to Fe ions, stimulation of cell viability/proliferation occurred in the first 2 weeks of culture and maximal values were observed around day 14 (Fe$^{0}$ caused the highest MTT reduction stimulation). Cultures exposed to Cr ions showed an increase in cell proliferation during the incubation time tested, although stimulation of MTT reduction was more significant in the first week (maximal levels were observed around day 7); stimulation was higher in the presence of Cr$^{0}$. In the presence of Ni ions, cultures showed somewhat mixed results, peak levels at day 7 (Ni$^{0}$), between days 14 and 21 (Ni$^{+}$) and variable effects (Ni$^{0}$). The effects of metal ions on MTT reduction appear to be more pronounced at the lower concentrations tested.

Fig. 2 shows results concerning the percentage alteration in MTT reduction for SS$^{0}$, Fe$^{0}$, Cr$^{0}$ and Ni$^{0}$ relative to the control (Fig. 2a) and for Fe$^{0}$, Cr$^{0}$ and Ni$^{0}$ over SS$^{0}$ samples (Fig. 2b). Results show that the effects of SS$^{0}$ and the corresponding metal ions in cell proliferation differ in terms of quantitative response (values similar to those observed in control medium in the presence of SS$^{0}$ solution and significantly higher in the presence of the...
Fig. 1. Percentage alteration in the MTT assay relative to the control. Effects of the three concentrations of: (a) the SS corrosion products; (b) Fe; (c) Cr; (d) Ni. *Significant differences ($P < 0.05$) between groups of metal exposed cells and control cells.
metal ions) and also in pattern of variation (trend for an increase towards the end of the culture with SS0 and stimulation more evident during the first 2 weeks of culture with the metal ions).

Light microscope observation of osteoblast-like cells showed that by day 7 all cultures (control and treated) were confluent (Fig. 3a), and by day 14 the cell layer began to detach from the surface culture forming a mass of roll tissue (Fig. 3b). From this aggregation new cells were proliferating towards the cell dish (Fig. 3c). The formation of such a mass of roll tissue has been observed quite often not only in our laboratory but also elsewhere [29]. These results are in agreement with Vrouwenfelder et al. [29] who have reported that 'after confluence it is often observed that the formed cell layer detach spontaneously from the underlying (non-relative) substrate' and/or since after confluence, cells grew forming multilayers and cells attached to the bottom of the plate may begin to die (and detach) due to the lack of nutrients and senescence.

3.2. Alkaline phosphatase activity

ALP activity has been routinely used in in vitro experiments as a relative marker of osteoblast differentiation. Osteoblast-like cells were identified on tissue culture dishes by showing a positive staining reaction for alkaline phosphatase (ALP) in histochemical assays carried out in cultures grown in the absence or in the presence of SS−, SS0 and SS+ solutions, Fe2+, Fe0 and Fe+ solutions, Cr+, Cr0 and Cr+ solutions or Ni−, Ni0 and Ni+ solutions. Similarly, to estimate the production of ALP, osteoblast-like cells were cultured in control medium or exposed to the above metallic solutions and were analysed weekly by biochemical means (see Materials and Methods). Results are shown in Figs. 4 and 5.

Fig. 4 shows results concerning dose- and time-dependent effects of alloy and metal solutions of ALP activity, relative to the control. Fig. 5 compares the effects of SS0, Fe0, Cr0 and Ni0 over control culture (Fig. 5a) and the effects of Fe0, Cr0 and Ni0 over SS0 treated cultures.
(Figs. 4a and 5a) than the corresponding metal ions (Figs. 4b–d and 5a), although in the presence of SS$^0$ and SS$^-$, a trend for ALP activity inhibition was observed, with an evident decrease in the levels of the enzyme at day 14. At days 7 and 21, Fe and Cr solutions caused significant increases in ALP activity. However, as shown before, cell proliferation was also stimulated in the presence of these solutions (Fig. 1). It is interesting to note that the percentage of simulation in MTT reduction was, in general, higher than the percentage of increase in ALP activity in the presence of these metal ions compared with control cultures (Figs. 1 and 4). Ni solutions had no effect in ALP activity, despite significant increase in cell proliferation observed in the presence of this ion. These results suggest that metal solutions appear to stimulate proliferation of a cell population with lower producing ALP ability than that growing in control conditions; this effect appears to be maximal in the presence of Ni solutions. SS corrosion products show a trend for a similar effect, although to a much lower extent (low stimulation in MTT reduction and low inhibition in ALP activity; Figs. 1a and 4a).

Results concerning ALP activity in normal and treated cultures do support the general conclusion that the solutions tested can affect the levels and temporal expression of ALP in rabbit osteoblast-like cell cultures.

Histochemical assays for calcium and phosphate deposits showed that the mineralization process was retarded in all cultures exposed to metallic solutions. Intensity of the staining was clearly lower, especially in cultures treated with Cr and Ni solutions.

Studies on the osteogenic cell system support the concept that osteoprogenitor cells differentiate into pre-osteoblasts, mature osteoblasts and then into osteocytes in discrete phases [30–34]. ALP activity is present when cells become recognized as pure-osteoblast and osteoblast (but it is absent from the osteocyte) and it is generally accepted that as the specific activity of ALP in a population of bone cells increases there is a corresponding shift to a more differentiated state [11, 16, 17, 30–34]. ALP appears to play a crucial role in the initiation of matrix mineralization and, after this, expression of the enzyme is down-regulated [15, 16, 17–34].

In this study, in control conditions, ALP attained maximal levels around day 14 and formation of mineral deposits occurred following the maximal ALP activity (after day 14). Synthesis of ALP by osteoblast-like cells appears to decrease in the presence of the metal solutions (effect suggested by analysing the results concerning percentage of alteration in cell viability/proliferation and in ALP activity in control and treated cultures). In addition, the significant inhibition in ALP activity observed in cultures treated with the metal solutions around day 14 suggests the possibility of an impairment in the mineralization process in these experimental situations. Decreased histochemical staining for calcium and

Fig. 3. Light microscope photographs of cell cultures showing: (a) cells in confluency ×4; (b) the mass of roll tissue formed by day 14 ×4; (c) new cells proliferating from the mass of roll tissue ×20.

(Fig. 5b). The solutions tested produced mixed results on alkaline phosphatase activity of osteoblast-like cells (Figs. 4 and 5). A common effect was a significant decrease in ALP activity during the second week of culture and, at day 14, levels of the enzyme were very low compared to the control. It is interesting to note that in this culture system and in control conditions, ALP is most rapidly produced around day 14. Stainless steel slurry caused significantly lower effects in ALP activity
Fig. 4. Percentage alteration in the ALP assay relative to the control. Effects of the three concentrations of: (a) the SS corrosion products; (b) Fe; (c) Cr; (d) Ni. *Significant differences ($P < 0.05$) between groups of metal exposed cells and control cells.
phosphates deposits observed in metal ion-treated cultures also supports this view.

Differences in effects between corrosion products from metal alloys and single metals, concerning proliferation and function of fibroblast and osteoblast-like cell cultures, have been reported previously [6, 7, 28] and should be a matter of further investigation in order to clarify the interaction of metal ions with biological systems.

4. Conclusions

Under the experimental conditions described, stainless steel corrosion products and the corresponding metal ions affect proliferation and function of osteoblast-like cell cultures. Results concerning cell viability/proliferation, ALP activity and histochemical identification of mineral deposits in control and treated cultures suggest a decrease in the expression of the osteoblast phenotype in the presence of ion and alloy solutions. Stainless steel corrosion products elicited slight effects but the corresponding metal ions (Fe, Cr and Ni) produced pronounced effects on the osteoblast phenotype, namely an alteration in the levels and temporal expression of ALP and lower and retarded tissue mineralization ability. Caution should be taken when comparing biological effects caused by separate metal salt solutions and those from metallic solutions derived from electrochemical dissolution of an alloy.

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References


