The use of rat, rabbit or human bone marrow derived cells for cytocompatibility evaluation of metallic elements

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Rat, rabbit and human bone marrow cells were cultured according to the method previously reported for cells of rat origin [1] and were exposed, or not (control), to corrosion products of a Co–Cr orthopaedic alloy as well as to metal salts containing Co^{2+}, Cr^{3+} and Cr^{6+}. Cells were cultured for 21 days and analysed for the following biochemical parameters: intracellular MTT reduction (i.e. cell viability/proliferation), alkaline phosphatase (ALP) activity and protein production. Morphological observations included both histochemistry (detection of ALP-positive cells, calcium and phosphate deposits) and scanning electron microscopy (SEM). Control cultures of rat and rabbit cells showed higher proliferation rates than human cells at the start of culture, but they all reached similar values on day 21. Protein production was parallel to cell proliferation. In contrast, ALP activity of rat cultures was much stronger than rabbit or human cultures. All cell types were able to develop the osteogenic phenotype *in vitro*.

Co–Cr extract caused inhibitory effects on cell viability, on ALP activity and, to a lower extent, on protein production of all rat, rabbit and human cell cultures. Compared to rat and rabbit cultures, human cultures were the most sensitive to metal ions exposure.

1. Introduction

Animal experimentation has been widely used to obtain information on biological responses to implants but it requires a huge number of animals in order to obtain reliable significance of results. Furthermore, results obtained in such *in vivo* models are difficult to interpret because there are so many and so diverse factors acting simultaneously in the body that the dose–response effect can be masked and misinterpreted. In contrast, cell cultures, which have been developed more recently, are ideal systems for dose–effect quantification of biomaterials degradation products and are now an important tool for biomaterials evaluation [2]. Thus, bone replacing materials and/or their degradation products must be tested on osteogenic cell cultures in order to examine the specific cellular response.

In the last decade, several methods to culture osteoblast-like cells have been reported but most of them lack the ability to mineralize *in vitro*. Cultures exhibiting the osteogenic phenotype, *i.e.* high intracellular alkaline phosphatase activity, synthesis of collagen type I and matrix mineralization, have been obtained from foetal or neonatal rodent [3–6] and chick [7, 8] calvaria; rodent [9–13] and human [14, 15] mandibular and alveolar bone; human skeletal tissues [16–18]; and rodent [1, 19, 20] and human [21, 22] bone marrow.

The method originally reported by Maniatopoulos *et al.* [1] for rat bone marrow derived cells was used in the present study and further adapted to rabbit and human bone marrow cells. The aim of this work was first, to compare the ability of cells from different species to develop into bone-like tissue; and, second, to examine the behaviour of rat, rabbit and human osteogenic cells when exposed to the corrosion products of a Co–Cr orthopaedic alloy.

2. Materials and methods

2.1. Metallic solutions

Accelerated corrosion of a Co–Cr–Mo (composition in wt%: Co (balance), Cr(28), Mo(5.5), Ni(1), Si(0.95),

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Fe(0.7), Mn(0.65) and C(0.25)) orthopaedic alloy was performed by chronoamperometric methods [23] in a NaCl 0.15 M electrolyte. Metallic contents in solution were determined in a Pye Unicam SP9 atomic absorption spectrophotometer with an air–acetylene flame using specific lamps for each element. The extract containing the corrosion products was then adjusted to a pH of 7.4 and sterilized in an autoclave. The procedure was the same with $CoCl_2 \cdot 6H_2O$, $CrCl_3 \cdot 6H_2O$ and Na_2CrO_4 solutions prepared at Co and Cr concentrations equal to those in the extract.

2.2. Cell culture

Primary cultures of osteoblast-like cells were established using bone marrow suspensions from rat, rabbit and human origin. For the tests, cells of the third subculture were seeded at 10^4 cells/cm^2 into multiwell culture plates and grown in α-minimal essential medium (Sigma) supplemented with 10% foetal bovine serum (Sigma), 2.5 μg/ml fungizone (Gibco), 50 μg/ml gentamicin (Gibco), 10^{-8} M dexamethasone (Sigma), 10 mM β-glycerophosphate (Sigma) and 50 μg/ml ascorbic acid (Sigma). The four above-mentioned metallic solutions were properly diluted in cell culture medium to attain 0.75 ppm in Co and/or 0.4 ppm in Cr and were tested in relation to a control consisting of the culture medium with only NaCl 0.15 M. Cultures were incubated for 7, 14 and 21 days at 37 °C in a humidified atmosphere of 5% Co_2. The media were changed twice a week.

2.3. Biochemical assays

Osteoblasts viability and proliferation was quantified by studying the cellular reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to a purple formazan product. Cells (eight wells for each situation) were incubated for 4 h with 0.5 mg of MTT per ml of culture medium. Afterwards, media were decanted, formazan salts were dissolved with 100 μl of dimethylsulphoxide and the absorbance was read at 600 nm.

For the measurement of alkaline phosphatase activity and of the total protein content, cultures (three wells for each case) were washed twice with phosphate-buffered solution (PBS) and stored at − 20 °C. Later, PBS with Triton X-100 (0.05% v/v) was added to the cultures, the cells were removed from the well bottoms by scraping and the suspension obtained was sonified for 10 s. Samples were then kept again at − 20 °C until further processing.

The total protein content was estimated by the Lowry method [24]. The quantities of all the involved reagents were adapted for 40 μl protein-containing samples in the rat and human cases, and for 250 μl samples in the rabbit situation. Absorbance was read at 600 nm and bovine serum albumin was used for standards preparation.

ALP activity was evaluated based on the transformation of p-nitrophenylphosphate into p-nitrophenol at 37 °C and pH = 10.3. The substrate (100 μl of a 20 mM solution) was added to 100 μl of each sample and the reaction was stopped by addition of 500 μl of NaOH 0.2 M after 30 min for rabbit cells and 15 min for rat and human cells. Optical density was measured at 405 nm and compared with the values of a series of p-nitrophenol standards.

2.4. Scanning electron microscopy

Cultures fixed with 1.5% glutaraldehyde (in 0.14 M sodium cacodylate buffer, pH = 7.4) for 10 min were dehydrated through a graded series of ethanol and critically point dried from CO_2 in a Balzers model CPD 020. Then, a layer of gold was sputter coated (in a Jeol JFC-1100) onto the specimens and they were examined in a Jeol JSM-6301F microscope equipped with an X-ray microanalysis capability Voyager XRMA system, Noran Instruments.

2.5. Histochemical staining techniques

Fixed cultures (as for SEM) were stained for ALP as well as for phosphorous and calcium deposits detection.

ALP: a substrate solution (prepared in Tris buffer, pH = 10) 2 mg/ml in Na-α-naphtyl phosphate and in fast blue RR salt was added to cells and left to actuate for 1 h in the dark.

Phosphates (Von Kóssa method): 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 then cultures were washed again.

Calcium: cultures were covered with a 1% S alizarin sodium sulfonate solution (0.028% v/v in NH_4OH), pH = 6.4, for 2 min and then rinsed with water and ethanol (0.01% v/v in HCl).

Stained samples were examined in an Olympus BH-2 microscope. Positive reactions were brown, black and red stained for ALP, phosphates and calcium, respectively.

2.6. Statistical analysis

Results are presented as mean values with 95% confidence intervals calculated using Student's *t*-parameter.

The Student's *t*-test was applied to determine the statistical significance of the differences between groups of results: *p* values lower than 0.05 were considered significant.

3. Results and discussion

3.1. Biochemical parameters

Bone marrow cells (third subculture) of rat, rabbit and human origin were cultured for 21 days and were weekly analysed for cell viability/proliferation (by the MTT assay), ALP activity (by detecting p-nitrophenol production) and protein contents (by the Lowry method).

3.1.1. Cell viability/proliferation

Rat and rabbit cells showed a high proliferation rate from day 7 to day 14, which decreased from day 14 to

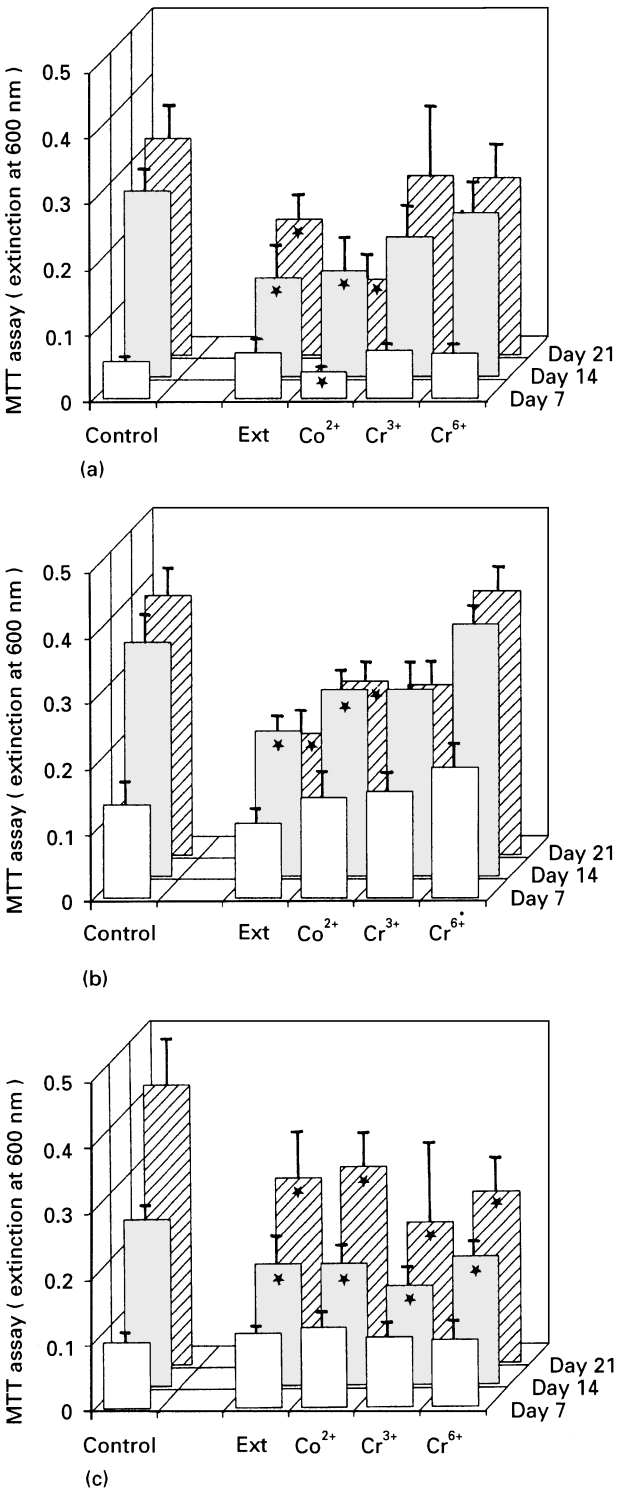

Figure 1 Cell viability estimated by the MTT assay. Effects over 21 days of the Co–Cr extract, Co^{2+}, Cr^{3+} and Cr^{6+} solutions for the rat (a), rabbit (b) and human (c) cells are shown. *Significant differences ($p < 0.05$) between groups of metal exposed cells and control cells.

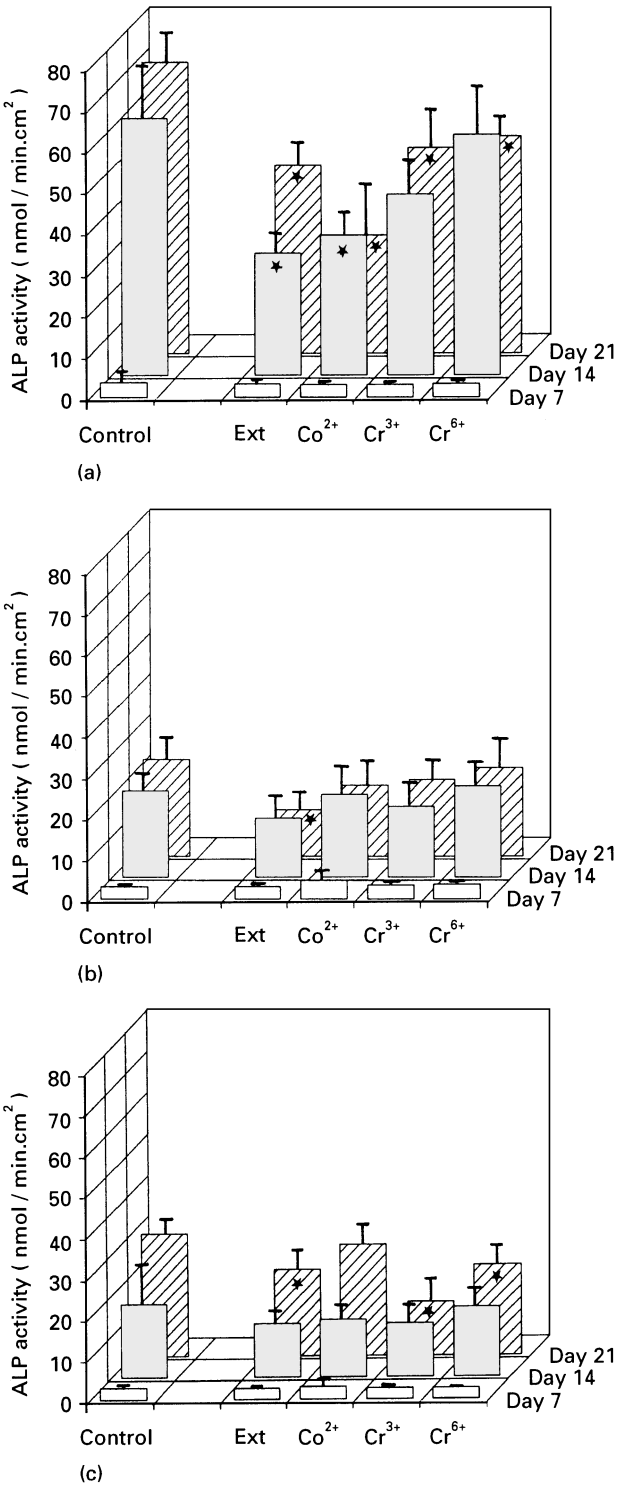

Figure 2 ALP activity. Effects over 21 days of the Co–Cr extract, Co^{2+}, Cr^{3+} and Cr^{6+} solutions for the (a) rat, (b) rabbit and (c) human cells are shown. *Significant differences ($p < 0.05$) between groups of metal exposed cells and control cells.

21 (Fig. 1a and 1b). In contrast, human cells presented a steady growth up to day 21 (Fig. 1c).

The exposure of cells to Co–Cr corrosion products caused inhibition of the cell proliferation in all types of cells after day 14 (Fig. 1). Similarly, Co^{2+} inhibited the proliferation of rat, rabbit and human cells. In contrast, Cr^{3+} and Cr^{6+} showed no effect on rat (Fig. 1a) and rabbit (Fig. 1b) cells, causing however significant inhibition of human cell growth (Fig. 1c). These results indicate that the Co–Cr extract and Co^{2+} are the most effective solutions in causing cell growth inhibition, and that human cells are more sensitive than rat and rabbit cells.

3.1.2. ALP activity

ALP activity of rat cultures was much stronger (on day 21: (71 ± 7 nmol/min cm^2) than rabbit (23 ± 5 nmol/min cm^2) or human (30 ± 3 nmol/min cm^2) cultures (Fig. 2). Since the number of cells plated

(10^4 cells/cm^2) on day 0 was the same for the three types of cultures, and the viability of cells was similar in all cultures on day 21 (Fig. 1), the higher ALP activity in rat cultures indicates that this culture was richer in osteoblasts than rabbit and human cultures. These results are not surprising since the method used in the present study was originally developed for rat cultures [1]. Perhaps some modifications in the experimental conditions could improve the enrichment of osteoblasts in rabbit or human cultures.

The exposure of cells to Co–Cr corrosion products caused significant inhibition of rat ALP activity on day 14 and 21, whereas rabbit and human cells were significantly affected just on day 21 (Fig. 2). Only the ALP activity of rat cells was affected by Co^{2+}, on day 14 and 21. Rat and human cells, but not rabbit cells, showed a significant decrease in ALP activity in the presence of Cr^{3+} and Cr^{6+}, on day 21 only. These results show that rabbit cells are less sensitive to the effects of metal ions as far as ALP activity is concerned.

3.1.3. Protein contents

Total protein contents in control cultures of all cellular types (Fig. 3) present a similar pattern to the one of cell viability (Fig. 1). Co–Cr extract caused inhibition of protein production in all cell types on day 21 (Fig. 3). Co^{2+} inhibited protein production in both rat and human cell cultures, on day 21, but not in rabbit cell cultures. Cr^{3+} inhibited both rabbit and human cells on day 21, and Cr^{6+} had no effects on any culture. Overall, the effects of metal ions on protein production (Fig. 3) were not as evident as on viability (Fig. 1) and ALP activity (Fig. 2).

3.2. Cell morphology and histochemistry

Morphological observation of cell cultures showed that rat, rabbit and human cells grew in clusters of cells, forming interconnected multilayered islands as originally reported by Maniatopoulos *et al.* [1]. Likewise, nodule formation was observed at 1 week culture and the polygonal cells at the periphery showed the osteoblast phenotype as they exhibited intense ALP activity by histochemistry.

At 2 weeks onward, extensive differences could be observed between the development of rat, rabbit and human osteogenic cultures. Mineralized nodules were clearly macroscopically identified as large, intense and distinct spots, after histochemical staining of rat cultures with alizarin red staining (for calcium) and Von Kóssa method (for phosphates). In rabbit cultures the nodules of mineralization were much smaller and weaker although distributed evenly through the cell culture. The nodules of human cell cultures were as disperse as in rabbit cultures, but strongly stained. The intensity of mineralization detected by such histochemistry assays is in accordance with biochemical assays, which showed that in control cultures ALP activity was stronger in rat, followed by human and rabbit cultures (Fig. 2). The correlation between ALP activity and mineralization is in agreement with previous studies demonstrating that ALP plays a main role in the initiation of mineralization [25].

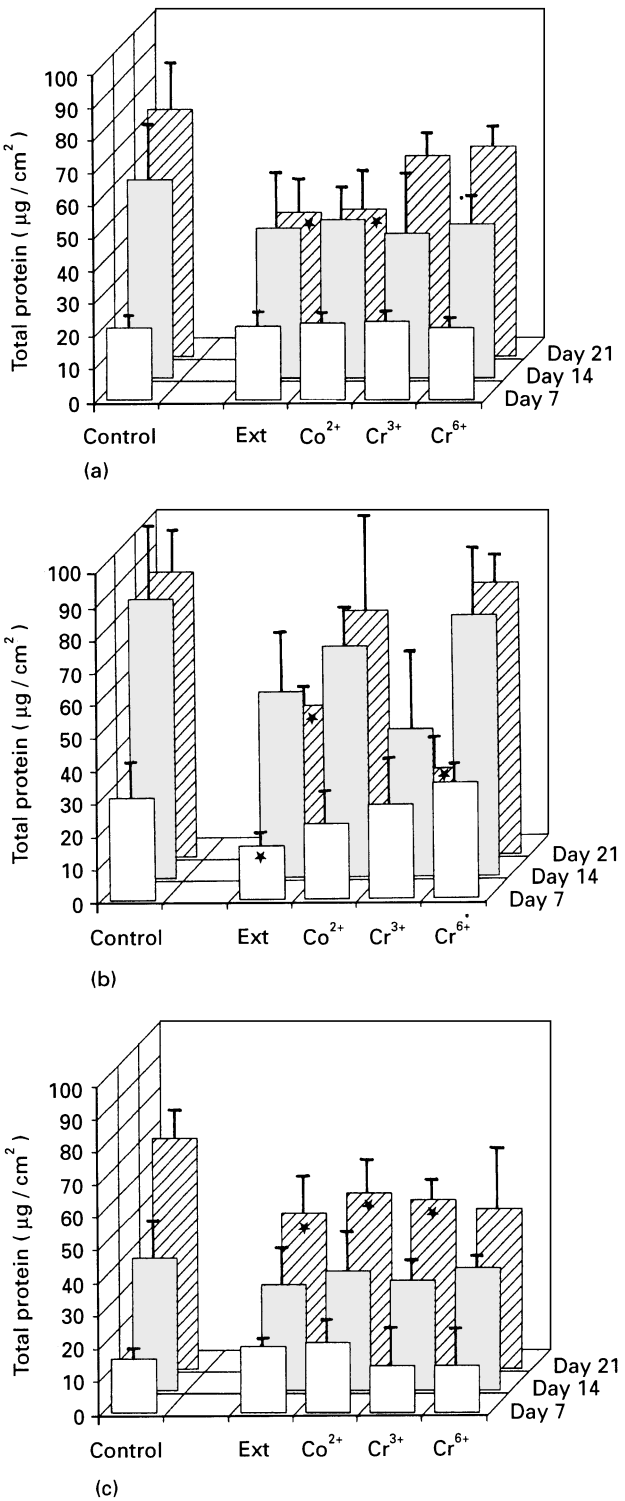

Figure 3 Total protein contents. Effects over 21 days of the Co–Cr extract, Co^{2+}, Cr^{3+} and Cr^{6+} solutions for the (a) rat, (b) rabbit and (c) human cells are shown. *Significant differences ($p < 0.05$) between groups of metal exposed cells and control cells.

Scanning electron microscopy of two-week-old rat, rabbit and human cultures further revealed that the top layer of the nodules was formed of large polygonal cells laying on a vast extracellular matrix composed of a fibrillar network, which is mostly composed of collagen, according to previous *in vitro* studies using either rat [26, 27] or human [14] bone marrow cells.

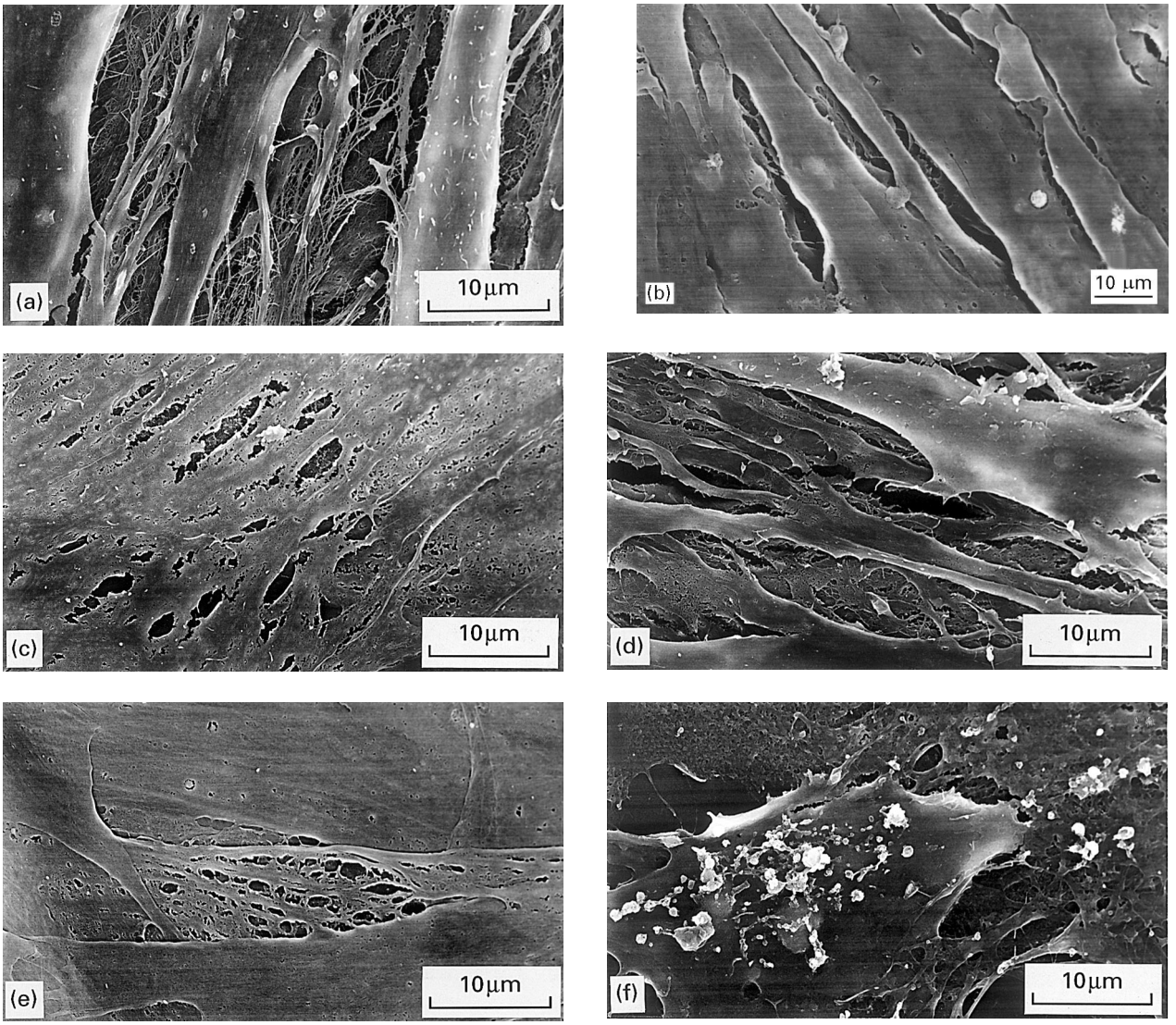

Figure 4 Scanning electron micrographs of 14-days-old osteogenic cultures derived from rat (a, d), rabbit (b, e) and human (c, f) bone marrow, and cultured in the absence (a–c) or in the presence (d–f) of Co–Cr corrosion products.

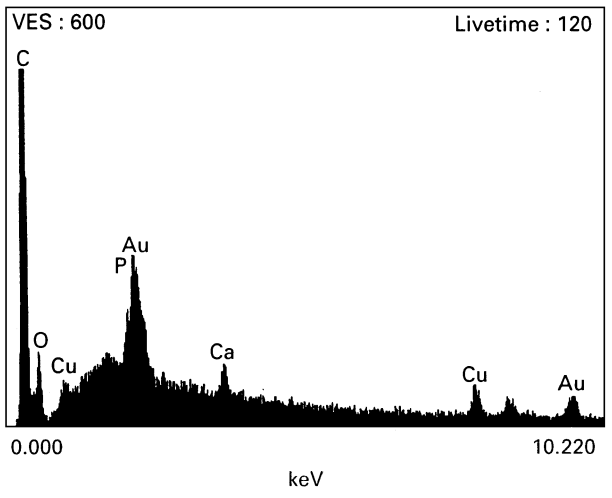

Figure 5 X-ray energy spectrum of mineralized areas showing the presence of phosphorous (P) and calcium (Ca). The carbon (C) and the oxygen (O) peaks are due to the biological material and the flask substrate, the copper (Cu) peak is due to the sample support and the gold (Au) peak to the sample coating.

Rat cells were connected to each other but showed large extracellular spaces (Fig. 4a), rabbit cells were much closer to each other (Fig. 4b) and human cells formed a continuous sheet, it being difficult to distinguish individual cells (Fig. 4c). However, fibres in the extracellular matrix could be observed below this cell layer beyond some cracks in the tissue. Calcium and phosphorous deposits in the extracellular matrix could be detected by an energy dispersive X-ray analyser in rat, rabbit and human samples as is exemplified in Fig. 5.

Cultures exposed to corrosion products of a Co–Cr alloy caused no evident effects on tissue organization as far as rat (Fig. 4d) and rabbit (Fig. 4e) cell cultures were concerned. In contrast, such corrosion products altered the morphology of human osteogenic cultures, which lost their smooth surface, and some dead cells could be observed in peripheral areas of the nodules (Fig. 4f).

4. Conclusions

Although the method used in this study was the one originally described by Maniatopoulos *et al.* [1] for rat bone marrow cells, rabbit and human cells were also able to develop the osteogenic phenotype since all cultures were ALP-positive (both histochemically and

biochemically), synthesized protein (which was shown to exhibit a fibrillar matrix) and mineralized.

Taken together, the results of the present work show that the Co–Cr extract was the metal solution causing inhibitory effects on cell viability, ALP activity and protein production on all rat, rabbit and human cells. Protein production was the biochemical parameter least affected by metal ions. Human cells were the most sensitive to metal ions exposure.

Whenever possible, cytocompatibility evaluation of biomaterials or their degradation products should be carried out on human cells as this *in vitro* model, compared to rat or rabbit, is closer to the clinical situation.

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