Cell death induced by metal ions: necrosis or apoptosis?

D. GRANCHI, E. CENNI, G. CIAPETTI, L. SAVARINO, S. STEA, S. GAMBERINI, A. GORI, A. PIZZOFERRATO
Laboratory for Biocompatibility Research on Implant Materials, Istituti Ortopedici Rizzoli, via di Barbiano 1/10, 40138, Bologna, Italy

We have evaluated if the cytotoxic effects of metals released from implants are due to necrosis or apoptosis. Peripheral blood mononuclear cells were exposed to different concentrations of chromium, nickel and cobalt extracts and the characteristics of both apoptosis and necrosis were evaluated by flow-cytometry at different culture endpoints. In order to define the prevalence of apoptosis or necrosis, the ratio cell death/apoptosis was calculated. A ratio of $\leqslant 1$ means the prevalence of apoptotic events; a ratio >1 indicates the acute toxicity of the tested substance (necrosis). The extracts of chromium, cobalt and nickel had a cytotoxic effect on the mononuclear cells; high concentrations of cobalt and nickel produced cell necrosis, whereas by lowering the extract concentration apoptotic phenomena were observed. High chromium concentrations can induce cell death by apoptosis. Our data suggest that when large amounts of nickel and cobalt are released from implanted metal devices, necrosis is produced and consequently a strong inflammatory tissue reaction is likely to occur. The release of either chromium or limited amounts of nickel and cobalt induces toxicity characterized by apoptotic phenomena, which allows an adaptation of the tissue to the implant.

1. Introduction

The release of corrosion products from implanted metals, mainly chromium, cobalt, nickel and titanium, has been associated with clinical complications such as adverse tissue reactions, promotion of infection and metal sensitivity. Local adverse reactions to metal products range from the inhibition of bone ingrowth in uncemented femoral stems [1] to major osteolysis around loose components [2]. In our previous experiments we have observed that metal extracts showed severe toxicity on lymphocytes cultured *in vitro*, with the exact degree of the effect depending on the ion concentration and the duration of exposure of the cells to the metal [3].

Nucleated eukaryotic cells die in two distinct modes: necrosis and apoptosis [4]. Severe hypoxia, lytic viral infections, complement attack, hyperthermia and exposure to toxic chemicals can all produce the degenerative phenomena of necrosis, which affects plasma membrane integrity and induces morphological changes [5].

Apoptosis or "programmed cell death" plays an important role in the physiologic turnover of normal cells, for the maintenance of tissue homeostasis, and follows exposure of the cells to some toxins. Cells undergoing apoptosis display a characteristic pattern of structural changes in the nucleus and cytoplasm, including rapid blebbing of the plasma membrane and fragmentation of DNA into internucleosomal fragments of 180–200 bp. When such DNA is analysed by agarose gel electrophoresis it generates the characteristic "ladder" pattern of discontinuous DNA fragments.

In toxicological studies it is fundamental to recognize the first signals of apoptosis or necrosis induced by cytotoxic stimuli. The apoptotic death is circumscribed to the involved cells, whereas the necrosis is accompanied by cell lysis, release of proteolytic enzymes and other products, which induce phlogosis and injure the surrounding tissue [4].

Flow cytometry can be applied to differentiate between the two modes of cell death, and several cytometric methods have been recently described for identifying cells undergoing DNA fragmentation [6]. The following parameters were found useful to distinguish apoptotic from necrotic cells: (a) unfixed dead cells lose plasma membrane integrity, and the damage is probed by the uptake of propidium iodide (PI); that occurs rapidly in necrotic cells, while it is a late event in apoptosis; (b) ethanol fixed apoptotic cells have a decrease in the stainability with specific fluorochromes, as a result of the loss of DNA fragments out of the cells.

The main goal of this study was to gain information into whether or not the cytotoxic effects of the metals released from orthopaedic implants are due to necrosis or apoptosis. Extracts of chromium, nickel and cobalt have been tested, because they are largely employed in the manufacture of both dental and orthopaedic implants, and their corrosion has been associated

0957–4530

with clinical complications, such as adverse tissue reactions and the loosening of prosthesis components [2, 7, 8].

Peripheral blood mononuclear cells were exposed to different concentrations of metal extracts and the flow-cytometric characteristics of both apoptosis and necrosis were evaluated at different culture endpoints.

2. Experimental procedures

2.1. Materials and methods

2.1.1. Preparation of extracts

Commercially available chromium, cobalt and nickel powders were extracted (Goodfellows, Malverne, PA, USA). The particle sizes of the powders were similar being 45–60 μm, and the purity was over 99%, according to the manufacturer's description.

The powders were extracted in AIM-V (GIBCO, Middlesex, UK), according to the ISO standards (1g per 5 ml of medium for 5 days at 37 °C) [9]. The extract was centrifuged and filtered onto filter paper containing 0.22 μm sized pores in order to eliminate any particulate residual. The product was aliquoted and frozen at −20 °C. Any contamination risk was avoided by performing all the steps under a laminar air-flow cabinet. Graphite furnace atomic absorption spectrometry (GFAAS) (Unicam Model Solaar 939QZ, Cambridge, UK) was used to measure the concentration of ions in the extracts.

2.1.2. Peripheral blood mononuclear cells isolation

Heparin-collected human peripheral venous blood (10 ml) was diluted 1:2 with saline solution and layered onto a Ficoll-Hypaque density gradient to separate mononuclear cells (PBMC) from erythrocytes and granulocytes [10]. The collected PBMC were washed with physiological solution by centrifugation for 10 min at $350 \times g$ and resuspended at 1×10^6 ml^{-1} in AIM-V containing L-glutamine (2 mM) (ICN Biomedical, Milan, Italy).

The PBMC were plated 100 μl per well in 96-well flat bottomed plates (Costar, Cambridge, MA, USA) and 100 μl of extract was added to them. Before testing the extract was diluted between 100 and 3% (vol) in the culture medium. The final concentrations in the wells were 50, 25, 12.5, 6, 3 and 1.5%, by doubling dilution. Some wells had 100 μl of the culture medium added to them in order to control normal cell growth. The cultures were incubated for 24, 48 and 72 h at 37 °C in a 5% CO_2/95% air atmosphere.

2.3. Viability assay

After 24, 48 and 72 h of culture, 10 μl of phosphate buffered saline (PBS) containing 10 μg ml^{-1} propidium iodide (PI) (Sigma, St. Louis, MO) were added to each microplate well. After a 30 min incubation at 37 °C each sample was evaluated by flow cytometry. PI is excluded by cells that have plasma membrane integrity, but it stains necrotic cells that have a damaged membrane, by intercalation with the double-stranded DNA and RNA [3, 11]. The analysis was performed using a flow cytometer equipped with two lasers (Epics-Elite, Coulter, Hialeah, FI). A 488 nm emission wavelength was suitable for PI excitation.

2.4. Flow cytometric detection of apoptosis

At the culture endpoints cells were collected and fixed for 30 min at 4 °C in 1 ml 70% cold ethanol. For each determination not less than 1×10^6 cells were used. The cells were washed twice with saline solution and resuspended in 1 ml PBS containing Ribonuclease A (RNAse – 0.75 mg ml^{-1}) (Sigma, St. Louis, MO) and PI 1 μg ml^{-1}.

After 30 min the cells were analysed using a flow cytometer as previously described. The proportion of apoptotic cells is considered to be the percentage of hypodiploid events with DNA index ranging from 0.60 to 0.95 in respect to the diploid peak. The positive control of apoptosis was performed on 2×10^6 HL60 cells cultured in 20 ml of complete RPMI 1640 (ICN Biomedical, Milan, Italy) and treated with campthotecin (Sigma, St. Louis, MO) at the final concentration of 0.5 μM. The culture was incubated for 4 h at 37 °C in a 5% CO_2 atmosphere.

2.5. Electrophoretical detection of apoptosis

At the culture endpoints lymphocyte cultures and HL60 cells were collected and washed once with saline solution. The cells were then resuspended in 0.4 ml of (TrisHCl buffer 10 mM EDTA 1 mM pH 8.0) enhanced with 0.2% Triton 100 ×. The samples were placed on a clino-roller at 4 °C for 30 min, then centrifuged in a microfuge for 15 min at 4 °C. The supernatant was collected and transferred into clean tubes. 0.045 ml of NaCl 5 M and then 0.4 ml of 100% isopropyl alcohol (Carlo Erba, Milan, Italy) were added to each sample. The samples were then frozen at −20 °C for 18 h. Later on the samples were centrifuged in the microfuge for 15 min at + 4 °C. They were then resuspended in 0.08 ml of Tris HCl Buffer 10 mM-EDTA 0.5 M.

An agarose gel (Sigma) at 1.5% in Tris–Borate–EDTA 1x buffer was prepared and enriched with ethidium bromide. In the meantime, 0.02 ml of loading buffer enriched with RNAse (10 μg ml^{-1}) were added; the samples were incubated at 37 °C for 30 min. DNA Molecular Weight marker VI (Boehringer Mannheim Biochemica, Mannheim, Germany) was used as the molecular weight marker for electrophoresis since it forms bands for molecular weights ranging between 0.15–2.1 Kpb. Electrophoresis was performed at 36 V for 8–9 h. The positive control of apoptosis was performed on HL60 cells, as above (2.4)

2.6. Calculations

The ratio cell death/apoptosis was calculated for each experiment and for each metal dosage. A ratio $\leqslant 1$ means a prevalence of apoptotic events that induce cell death; a ratio > 1 indicates the acute toxicity of

the tested substance, which rapidly changes the cell structure (necrosis). The percentage of both dead and apoptotic cells, and the ratio between these values, were expressed as the arithmetic mean plus and minus the standard error of the mean (m $\pm$ SEM) of five separate experiments carried out in the same working conditions.

Statistical evaluation of the effects of extract dilution on the cell viability was made by analysis of variance (ANOVA), and Bonferroni Dunn's multiple comparison test was applied to detect specific differences between groups (StatView 4.5 for Macintosh, Abacus). Only p values $\leqslant 0.01$ were considered as statistically significant.

3. Results

The amount of ions released from the extracted metals were different, although the same method of extraction and the same particle size were chosen for the powders. The release of chromium was 0.114 ± 10 $\mu g\,ml^{-1}$, whereas the amounts of Co and Ni were 53.9 ± 0.6 and 51 ± 0.2 $\mu g\,ml^{-1}$, respectively.

The effectiveness of the cytofluorimetric method in detecting apoptosis was confirmed by means of electrophoresis on the agarose gel of the DNA of HL-60 cells treated with campthotecin 10 μM: when on agarose gel the characteristic "ladder" pattern of discontinuous DNA fragments is generated (Fig. 1), flow-cytometry revealed 43.5% of hypodiploid events (Fig. 2(a and b)).

The proportion of both dead and apoptotic cells is reported in Tables I and II, respectively. It can be observed that apoptotic cells always occur in the

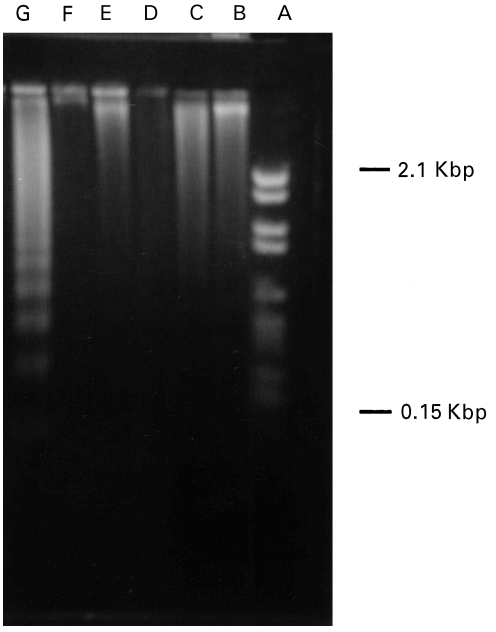

Figure 1 Agarose gel electrophoresis of DNA extracted after 72 h of culture under various experimental conditions. The micrograph represents the molecular weight markers (lane A), the untreated PBMC (lane B), PBMC treated with 50% of chromium extract (lane C), 50% of cobalt extract (lane D), 50% of nickel extract (lane E), the untreated HL-60 cells (lane F), HL-60 treated with 10 μm of campthotecin (lane G).

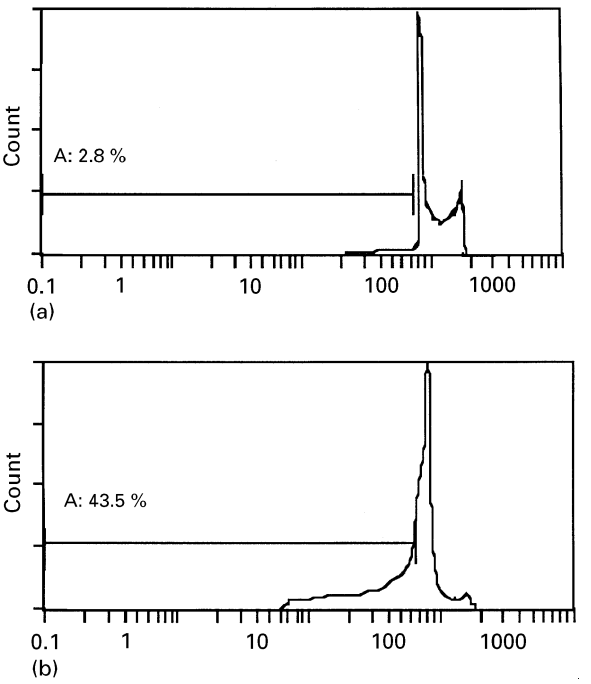

Figure 2 DNA content histograms of HL-60 cells (a) untreated and (b) treated with 10 μM of campthotecin. The linear gates indicates the hypodiploid events in the examined sample. (x axis = fluorescence intensity of PI on log scale.)

TABLE I Proportion of dead cells

	Cell death (%)		
	24 h	48 h	72 h
Control	2.7 ± 1	6.4 ± 1	9.3 ± 1
Chromium (%)			
50	4.7 ± 0^{b}	10.8 ± 1^{a}	18.2 ± 1^{a}
25	3.4 ± 1	9.5 ± 1^{b}	16.0 ± 1^{a}
12.5	3.6 ± 1	8.8 ± 1	16.5 ± 2^{a}
6	3.4 ± 1	9.1 ± 2	15.2 ± 2
3	3.0 ± 1	8.3 ± 1	13.2 ± 2
1.5	2.8 ± 1	7.3 ± 0.5	11.5 ± 2
Cobalt (%)			
50	10.8 ± 3^{a}	43.0 ± 2^{a}	76.7 ± 1^{a}
25	4.7 ± 1	22.3 ± 1^{a}	59.4 ± 1^{a}
12.5	4.1 ± 1	9.2 ± 2	24.8 ± 1^{a}
6	5.0 ± 2	8.4 ± 2	16.6 ± 1^{a}
3	3.4 ± 1	7.7 ± 2	16.5 ± 4^{a}
1.5	3.6 ± 1	7.0 ± 2	13.4 ± 1^{b}
Nickel (%)			
50	10.3 ± 1^{a}	19.8 ± 3^{a}	41.2 ± 3^{a}
25	6.3 ± 1^{a}	14.1 ± 2^{a}	25.0 ± 3^{a}
12.5	3.9 ± 0.5	10.8 ± 1^{a}	18.5 ± 1^{a}
6	2.9 ± 0.5	6.9 ± 1	14.4 ± 1^{b}
3	2.3 ± 0	4.8 ± 1	10.6 ± 1
1.5	2.2 ± 0	5.6 ± 1	8.9 ± 1

[a] $P \leqslant 0.001$

[b] $P \leqslant 0.01$

control cultures and also that the apoptosis percentage tends to increase in time, as does the percentage of dead cells. The ratio between dead cells and apoptotic cells (Table III) is always $\leqslant 1$, thus demonstrating that without the occurrence of exogenous factors apoptosis represents the prevailing mechanism determining the spontaneous death of PBMC in a culture.

TABLE II Proportion of apoptotic cells

	Apoptosis (%)		
	24 h	48 h	72 h
Control	12.6 ± 2	17.1 ± 2	18.8 ± 2
Chromium (%)			
50	17.0 ± 3	21.6 ± 1	26.3 ± 3
25	14.8 ± 3	18.5 ± 3	20.6 ± 3
12.5	12.7 ± 3	18.2 ± 4	24.2 ± 6
6	9.6 ± 5	20.5 ± 4	20.2 ± 2
3	9.8 ± 5	21.2 ± 4	21.6 ± 3
1.5	10.4 ± 5	20.3 ± 7	22.7 ± 4
Cobalt (%)			
50	17.4 ± 1	27.1 ± 2[a]	28.4 ± 3[a]
25	14.4 ± 2	21.3 ± 1	24.1 ± 2
12.5	14.1 ± 3	19.9 ± 2	21.9 ± 4
6	16.0 ± 5	19.0 ± 2	21.5 ± 3
3	13.7 ± 4	17.7 ± 4	19.3 ± 4
1.5	13.8 ± 4	18.8 ± 2	19.0 ± 4
Nickel (%)			
50	20.0 ± 2[a]	22.6 ± 2[a]	27.1 ± 3[a]
25	20.8 ± 3[a]	23.9 ± 1[a]	25.9 ± 2[a]
12.5	19.0 ± 5	21.3 ± 1	26.3 ± 1[a]
6	13.6 ± 3	19.7 ± 2	21.9 ± 4
3	15.7 ± 3	20.3 ± 3	18.6 ± 3
1.5	13.5 ± 0	17.8 ± 0	22.2 ± 2

[a] $P \leqslant 0.01$

TABLE III Ratio dead cells/apoptotic cells

	Cell death/Apoptosis		
	24 h	48 h	72 h
Control	0.2 ± 1	0.4 ± 1	0.5 ± 1
Chromium (%)			
50	0.3 ± 0.1	0.5 ± 0.1	0.7 ± 0.1
25	0.5 ± 0.3	0.6 ± 0.1	0.7 ± 0.1
12.5	0.5 ± 0.2	0.6 ± 0.1	0.7 ± 0.2
6	1.1 ± 0.5	0.5 ± 0.1	0.7 ± 0.1
3	1.1 ± 0.6	0.4 ± 0.1	0.7 ± 0.1
1.5	0.7 ± 0.3	0.6 ± 0.3	0.5 ± 0.1
Cobalt (%)			
50	0.7 ± 0.4	1.7 ± 0.3	2.9 ± 0.5
25	0.4 ± 0.2	1.1 ± 0.1	2.3 ± 0.2
12.5	0.5 ± 0.3	0.4 ± 0.1	0.9 ± 0.1
6	0.7 ± 0.5	0.4 ± 0.1	0.7 ± 0.1
3	0.4 ± 0.3	0.4 ± 0.1	0.7 ± 0.2
1.5	0.7 ± 0.6	0.3 ± 0.1	0.6 ± 0.1
Nickel (%)			
50	0.5 ± 0.1	0.9 ± 0.1	1.6 ± 0.1
25	0.3 ± 0.1	0.6 ± 0.1	1.0 ± 0.1
12.5	0.3 ± 0.1	0.6 ± 0.1	0.7 ± 0.1
6	0.2 ± 0.1	0.5 ± 0.1	0.7 ± 0.1
3	0.2 ± 0	0.4 ± 0.1	0.6 ± 0.1
1.5	0.2 ± 0	0.2 ± 0.1	0.4 ± 0.1

Chromium toxicity appeared after 24–48 h in the culture for the highest extract concentrations (50 and 25%) with a significant increase of cell death, and after 72 h in the extract concentrations ranging between 50–12.5%. The apoptotic cell percentage increased with respect to the control culture even though not significantly; when the cell death was significantly increased, the ratio-cell death/ apoptosis was <1.

Cobalt toxicity appeared after 24 h in the culture with a significant increase in the number of dead cells at the highest extract concentration. After 48 h the toxicity was evident for the 25% concentration, and after 72 h the extract was toxic down to a concentration of 3%. The apoptotic cell percentage increased significantly after 48 h to the 50% concentration. The ratio cell death/apoptosis was $\leqslant 1$ at 24 h; at 48–72 h it was >1 for the 50 and 25% concentrations whereas to the extract concentrations ranging between 12.5 –1.5% the ratio was $\leqslant 1$.

Nickel toxicity was observed after a 24 h culture with a significant increase in cell death for the 50 and 25 % concentrations. After 48–72 h toxicity was also observed for the 12.5% concentration. The apoptotic cell percentage increased significantly after a 24–48 h exposure to both the 50 and 25% extracts, and after 72 h with the 12.5% extract. After 24–48 h the ratio cell death/apoptosis was <1 for all the extract concentrations while after 72 h this ratio for the 50% concentration was >1, whereas for concentrations lower than this the ratio was $\leqslant 1$.

Fig. 3(a–f) displays some histograms representing both cell death and apoptosis in PBMC after 72 h of culture with the 50% metal extracts. The data obtained by flow cytometry were checked by measuring the DNA migration with electrophoresis on agarose gel (Fig. 1). HL-60 cells treated with 10 μM camphtotecin were used as the positive control. The DNA of untreated HL-60 cells does not migrate and shows a band close to the deposition area, whereas those incubated with campthotecin displayed the typical fragmentation of apoptic DNA (DNA ladder). The detectable bands had approximately the following molecular weights: 300, 453, 653 and 800 p.b. The DNA of the untreated PBMCs was concentrated near the deposition area with a weak migration smear. The DNA of the PBMCs treated with the chromium extract looked very similar even though the deposition band was less evident and some weak bands were observed. The DNA of the PBMCs treated with the cobalt extract was probably denaturated, because it did not appear near the deposition area nor did it migrate. The PBMCs treated with the nickel extract behaved in a similar manner to the chromium extract, but the DNA fragmentation bands were absent.

4. Discussion

The term apoptosis is used to describe a characteristic type of cell death induced by different environmental stimuli in various cell types [4]. In contrast with apoptosis, necrosis is an aspecific mechanism of cell death, which can be produced by high concentrations of toxic agents. The differentiation between cell death due to necrosis or apoptosis is very important in toxicology, as the biological significance of these two events is deeply different. One major distinction between apoptosis and necrosis *in vivo* is that complete elimination of apoptotic cells by phagocytes prevents

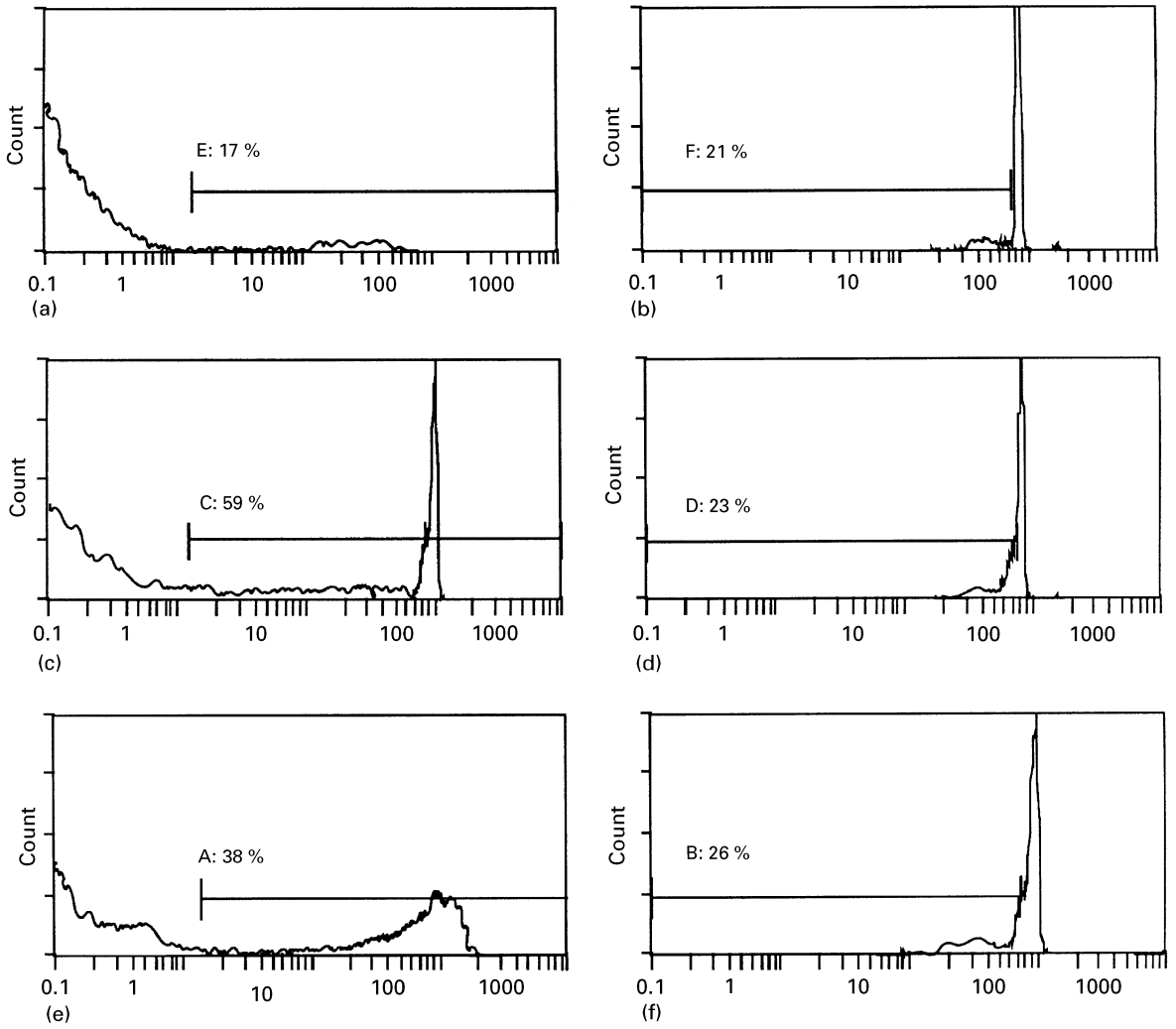

Figure 3 DNA stainability of un-fixed (a, c, e) and fixed (b, d, f) PBMC after 72 h of culture with 50% metal extracts. (a) Chromium cell death, (b) Chromium apoptosis, (c) Cobalt cell death, (d) Cobalt apoptosis, (e) Nickel cell death and (f) Nickel apoptosis. Propidium iodide (PI) stains unfixed cells when plasma membrane integrity is lost. The percentage of PI positive cells indicates the proportion of cell death, but it does not distinguish apoptosis or necrosis. PI stains all fixed cells and apoptotic cells can be differentiated for a decrease in the stainability as a result of loss of DNA fragments. The linear gates indicates the PI uptake of un-fixed cells (A-C-E) and the hypodiploid events in the examined sample (B-D-F). (x-axis = fluorescence intensity of PI on log scale.)

an inflammatory response, whereas necrosis is characterized by phenomena inducing phlogosis and injuries to the surrounding tissue.

In our study the cytotoxic effects of the extracts of chromium, nickel and cobalt were evaluated. These metals were chosen because they find significant use in the manufacture of both dental and orthopaedic implants, and their corrosion behaviour has been associated with clinical complications, such as adverse tissue reactions and loosening of prosthesis components. The toxicity of these metals for *in vitro* culture systems is known, but for the above mentioned reasons it seems useful to establish whether the toxic effects are due to apoptosis or necrosis. In the former case the tissue surrounding the prosthesis is more likely to adapt to the microenvironmental modifications, whereas in the latter case the inflammatory phenomena consequent to necrosis can induce a severe tissue reaction.

In our experimental design, peripheral blood mononuclear cells were exposed to different concentrations of metal extracts and the flow-cytometric characteristics of both apoptosis and necrosis were evaluated at different culture endpoints. Our data show that the cytofluorimetric evaluation results are more sensitive than the electrophoresis on agarose gel as previously reported in the literature [12]. This is because flow-cytometry is able to detect small percentages of apoptosis, which are not evident with electrophoresis.

The apoptosis percentage found in unexposed PBMCs tended to increase as time elapsed, as well as the percentage of dead cells stained by propidium iodide. The senescence of the cell culture and the decrease of cell growth factors in the medium are able to induce the programmed cell death of mononuclear cells [13].

The addition of the metal extract induced cell death. Chromium toxicity was shown after 48 h at the highest extract concentration, and after 72 h the toxic effect was observed at extract concentrations ranging between 50–12.5%. Cobalt toxicity appeared after 24 h with the highest extract concentration: it increased at the successive endpoints and after 72 h the toxicity was evident down to a concentration of 1.5%. The highest nickel concentrations resulted in significant

cytotoxicity after 24 h whilst after 48–72 h toxicity was evident even for the 12.5% concentration. These data confirm our previous observations that the different toxicity behaviours of metal extracts can be ascribed to the type of metal, to its concentration and to the time of contact with the cells. Actually the release of cobalt and nickel ions into the extraction fluid is much higher than that of the chromium ions [3].

The apoptosis rate was increased and the difference with respect to the control culture was statistically significant for the highest concentration of cobalt extract at 48–72 h, for the 50 and 25% nickel extracts at 24–48 h, and for the 12.5% nickel extract at 72 h.

In vivo the apoptotic cells are removed rapidly by phagocytes, while in an "*in vitro*" system they remain and lose cell membrane integrity and hence assume the flow-cytometric characteristics of necrotic cells. In this circumstance it is very difficult to establish whether cell death is due to necrosis or apoptosis. We have assumed that the ratio between cell death and apoptosis can be considered as a reliable index of the toxic effects of the metal extracts. When the ratio is $\leqslant 1$ it means that apoptotic events prevail on the dead cells. This means that the toxic effects of the examined substance are linked to apoptotic phenomena. A ratio > 1 indicates that the examined material behaves as a strong toxic substance, causing structural alterations.

The ratio for the chromium extracts were always < 1, that is in favour of the percentage of apoptotic cells. This shows that the significant cell death caused by the high chromium concentration extracts could be linked to apoptotic phenomena rather than to necrosis. These data are in agreement with reports in the literature that apoptosis is the mode of cell death of a CHO cell line treated with sodium chromate [14] and also that chromium is able to induce apoptosis in human lymphocytes exposed to PHA [15]. The apoptosis induced by chromium seems to be linked to the type of cell used in the experiment. Actually it has been reported that both the DNA pattern and the ultrastructure of L-929 cells and human gingival fibroblasts after exposure to chromium, revealed signs of necrosis but no signs of apoptosis [16].

With the 50% cobalt extract cell death was significantly increased after 24 h and the cell death/apoptosis ratio was < 1 whereas at 48–72 h both the 50 and 25% concentrations had ratios that were significantly higher than 1. A reasonable assumption to explain this behaviour is that the cobalt toxicity could be started by apoptotic phenomena, but not the strong toxicity of the high metal concentrations which becomes more evident with time. As the cobalt concentration decreases the toxicity of cobalt is still significant and the ratio of cell death/apoptosis in favour of the apoptotic cells.

The exposure to the nickel extract induced similar effects in that after a 24–48 h exposure to the 50 or 25% extracts the cell death was significantly increased and the ratio cell death/apoptosis is less than 1. The ratio becomes > 1 with the 50% concentration extract only after 72 h. This demonstrates that large concentrations of nickel are more able to induce structural changes in the cells. When the concentration decreases the cell death/apoptosis ratio also decreases because the toxic effect is probably due to apoptosis phenomena.

5. Conclusion

In conclusion our data show that extracts of chromium, cobalt, and nickel have a cytotoxic effect on isolated mononuclear cells; the toxic effect depends on the type of metal, on the extract concentration and on the time of contact between mononuclear cells and extract. High concentrations of cobalt and nickel seem to be able to produce cell necrosis phenomena, whereas at low concentrations apoptotic phenomena prevail. High chromium concentrations can induce cell death by apoptotic phenomena. These results allow us to put forward some hypotheses on the role of cobalt, chromium and nickel ions that can be released from an implant due to corrosion phenomena. The release of large amounts of nickel and cobalt could favour necrotic phenomena and the inflammatory reaction of the surrounding tissue, as it is observed in the histologic findings of loosened protheses [17]. The release of chromium or of limited amounts of cobalt and nickel could however be followed by toxic effects, characterized by apoptotic phenomena which are less serious from a biological viewpont, because they allow an adaptation of the tissue to the implant.

Acknowledgements

This research is partially supported by grants from the Istituti Ortopedici Rizzoli, 'Ricerca Corrente' and Finalized Project 'Citocompatibilità Quantitativa nella Valutazione dei Materiali Protesici in Chirurgia Ortopedica'.

References

1. S. D. COOK, R. L. BARRACK, and A. J. T. CLEMOW, *J. Bone Jt. Surg.* **76–B** (1994) 68.
2. F. F. BUECHEL, D. DRUCKER, M. JASTY, W. JIRANEK and W. H. HARRIS, *Clin. Orthop. Rel. Res.* **298** (1994) 202.
3. D. GRANCHI, G. CIAPETTI, L. SAVARINO, D. CAVEDAGNA, M. E. DONATI and A. PIZZOFERRATO. *J. Biomed. Mater. Res.* **31** (1996) 183.
4. L. D. TOMEI and F. O. COPE (eds), in "Current Commun. Cell and Mol. Biol.", Vol. 3 (Cold Spring Harbor, NY, 1991) p. 410.
5. A. BERNELLI-ZAZZERA, in "*Patologia Generale.*", G. M. Pontieri (ed.) (Piccin Nuova Libraria, Padova, 1987) p. 216.
6. Z. DARZYNKIEVICZ, S. BRUNO, G. del BINO, W. GORCZYCA, M. A. HOTZ, P. LASSOTA and F. TRAGANOS, *Cytometry* **13** (1992) 795.
7. D. R. HAYNES, S. D. ROGERS, S. HAY, M. J. PEARCY and D. W. HOWIE, *J. Bone Jt. Surg* **75-A** (1993) 825.
8. C. D. BROWN, M. D. LOCKSHIN, E. A. SALVATI and P. G. BULLOUGH, *ibid* **59-A** (1977) 164.
9. ISO 10993 "Biological testing of medical devices – Part 12: Sample preparation and reference materials", (1996).
10. A. BOYUM *Scand. J. Clin. Invest.* **97** (1968) 1.
11. Z. DARZYNKIEVICZ, in "*Flow cytometry and sorting*", M. R. Melamed, T. Lindmo, M. L. Mendelson (eds) (Wiley, New York, 1990) p. 291.
12. J. GONG, F. TRAGANOS and Z. DARZYNKIEVICZ. *Analy. Biochem.* **218** (1994) 314.

13. C. M. PAYNE, L. GLASSER, M. E. TISCHLER, D. WYCOF, D. CROMEY, R. FIEDERLEIN and O. BOHNERT, *Micros. Res. Tech.* **28** (1994) 327.
14. L. J. BLANKENSHIP, F. C. MANNING, J. M. ORENSTEIN and S. R. PATIERNO, *Toxicol. Appl. Pharmacol.* **126** (1994) 75.
15. R. RAJARAM, B. U. NAIR and T. RAMASAMI, *Biochem. Biophys. Res. Commun.* **16** (1995) 434.
16. A. SCHEDLE, P. SAMORAPOOMPICHIT, X. H. RAUSCH FAN, A. FRANZ, W. FUREDER, W. R. SPERR, W. SPERR, A. ELLINGER, R. SLAVICEK, G. BOLTZ and G. NITULESCU, *J. Dent. Res.* **74** (1995) 1513.
17. A. PIZZOFERRATO, G. CIAPETTI, S. STEA and A. TONI, *Clinical Mater.* **71** (1991) 51.

Received 30 October 1996
and accepted 7 April 1997