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Effect of cobalt and chromium ions on bcl-2, bax, caspase-3, and caspase-8 expression in human U937 macrophages

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Abstract

The bcl-2 and caspase families of proteins play a central role in the modulation of apoptosis. The purpose of this study was to analyze the effect of Co^{2+} and Cr^{3+} ions on the expression of bcl-2, bax, caspase-3 and caspase-8 to better understand the mechanisms leading to ion-induced apoptosis in macrophages. U937 human macrophages were exposed to Co^{2+} and Cr^{3+} ions. The expression of proteins was measured by Western blot while caspase activities were measured by colorimetric assay. Results show that Co^{2+} ions inhibited bcl-2 expression with significant effect ($p<0.05$) after 16 h and a maximal 52% inhibitory effect after 24 h. Co^{2+} stimulated bax expression with a significant stimulation ($p<0.05$) after 8 h and a maximal 1.75-fold increase after 16 h. Co^{2+} also stimulated the expression of the active fragment of caspase-3 as well as caspase-3 activity maximal increase after 24 h. Co^{2+} ions had no effect on caspase-8 expression or activity.

Cr^{3+} ions inhibited bcl-2 expression with significant effect ($p<0.05$) after 16 h and a maximal 43% inhibitory effect after 24 h. Cr^{3+} stimulated bax expression with significant stimulation ($p<0.01$) after 8 h and a maximal 2.25-fold increase after 24 h. Cr^{3+} ions also stimulated the expression of the active fragments of caspase-3 and -8, as well as the activities of both proteases. The effect of Cr^{3+} ions on the expression of both caspase active fragments was maximal after 16 h incubation. In conclusion, our results suggest that the modulation of the expression of proteins from the bcl-2 and the caspase families of proteins are implicated in the induction of macrophage apoptosis by Co^{2+} and Cr^{3+} ions.

Keywords: Apoptosis; Hip replacement; Prosthesis; Macrophage; Metal ions

1. Introduction

Wear particles leading to periprosthetic osteolysis and subsequent loosening is a critical process that limits the longevity of total hip arthroplasty (THA). Polyethylene particles have been the main culprit in initiating osteolysis [1,2]. Because of their potential for improved wear performance, there has been a revived interest in metal–metal bearings, made of cobalt–chromium–molybdenum alloys, as an alternative to the use of conventional metal–polyethylene bearings. However, metal ion toxicity remains a major cause for concern [3,4]. Indeed, there are multiple sources of metallic corrosion products in the periprosthetic environment, including wear particles, fretting at the head–neck interface, and the passive oxidation layer that covers the prosthesis.

Our laboratory demonstrated that both cobalt (Co^{2+}) and chromium (Cr^{3+}) ions induced macrophage cell death in a dose-dependent manner. The results also suggested that Co^{2+} and Cr^{3+} ions induced apoptosis via a pathway implicating caspase-3 [5,6]. Apoptosis is an active form of cell death that requires the participation of active cellular processes. Indeed, the interest in apoptosis lies in the fact that it is under positive and negative regulation through evolutionary conserved biochemical pathways and therefore offers specific targets for therapeutic intervention.

One of the first cell death-regulating genes to be identified was bcl-2, an anti-apoptotic gene. Subsequent

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to this discovery, a variety of genes exhibiting significant sequence similarity with bcl-2 have been identified. Some members of this family, such as bcl-2, function as cell-death suppressors, and other homologues, including bax, have powerful death promoting abilities. Although the molecular mechanisms by which these proteins exert their pro- and anti-apoptotic functions are not totally defined, it is well known that they play a pivotal role in apoptotic cell death [7,8]. With regard to periprosthetic osteolysis, the in situ analysis of interface membranes (IMs) from aseptically loosened implants showed that bax is expressed mainly in macrophages [9]. However, not all cells that express this apoptotic-promoting protein enter into apoptosis.

Other studies led to the isolation of a family of molecules that encode for cysteine proteases, namely the caspases. Although apoptosis seems to uniformly require the participation of caspases, the particular caspase pathways vary according to cell-type and stimulus [10]. Among these proteases, caspase-3 is probably best correlated with apoptosis and is commonly activated by numerous death signals and cleaves a variety of important cellular proteins [11]. We recently demonstrated that caspase-3 is activated both in in vivo IMs from loose THA [12] and in in vitro alumina ceramic-stimulated macrophages [13]. Because of its localization at or near the membrane, caspase-8 represents the most receptor-proximal caspase and induces a cascade of caspases, including caspase-3 [14]. We recently demonstrated the presence of caspase-8 active fragment in IMs from loose THAs whereas this fragment was absent from control tissues [12].

The purpose of this study was to analyze the effect of Co^{2+} and Cr^{3+} ions on the expression of bcl-2, bax, caspase-3, and caspase-8 to better understand the mechanisms leading to ion-induced apoptosis in macrophages.

2. Materials and methods

2.1. Cell culture

U937 human macrophages were cultured in suspension in low adhesion culture flasks for cell suspension (Sarstedt, St-Léonard, Que., Canada) in RPMI 1640 medium (Biomédia Canada, Drummondville, Que., Canada) supplemented with 5% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Macrophages were exposed, in suspension, to 0–10 ppm Co^{2+} ($CoCl_2$, Fisher Scientific, Ville St-Laurent, Que., Canada) and 0–500 ppm Cr^{3+} ($CrCl_3$, Sigma Chemicals, Oakville, Ont.) for 0–24 h at a concentration of 5×10^5 cells/ml of culture media. Macrophages alone served as negative control. Incubations were conducted at 37°C in a 5% CO_2 environment.

2.2. Protein expression

The expression of proteins was measured by Western blot as recently described [13], with minor modifications. Briefly, cells were lysed in 100 μl of caspase assay lysis buffer from BioSource (Nivelles, Belgium), proteins were separated on 4–20% acrylamide gels and transferred to nitrocellulose membrane. Blots were performed using specific antibodies (bcl-2 [♯MS-598] and bax [♯MS-714], NeoMarkers, Fremont, CA, USA—cleaved caspase-3 [♯2305-PC], Trevigen, Gaithersburg, MD, USA—cleaved caspase-8 [♯9748], New England BioLabs, Mississauga, Ont., Canada). Actin (♯MS-1295, NeoMarkers) expression served as an internal control for protein loading. Cleaved poly(ADP-ribose)polymerase (PARP) (♯44-698Z, BioSource) was used as a marker of apoptosis. Proteins were detected using *NEN Renaissance* chemiluminescence reagents (Perkin-Elmer, Boston, MA, USA). Protein expression was detected and analyzed using Bio-Rad VersaDoc equipped with a cooled CCD 12-bit camera.

2.3. Caspase activity

Caspase-3 and caspase-8 activities were measured by colorimetric assays (BioSource, Belgium) based on the recognition of specific amino acid sequences by these caspases. The activity was detected by the measurement at 405 nm of free *p*-nitroanilide (*p*-Na) release from the cleavage of the substrates by caspase-3 (*p*-Na-DEVD {Asp–Glu–Val–Asp}) and caspase-8 (*p*-Na-IETD {Ile–Glu–Thr–Asp}). Results are the mean ± SD of three experiments performed in duplicate.

2.4. Statistical analysis

Statistical significance was calculated using ANOVA followed by Fisher's PLSD comparison test. $p < 0.05$ was considered significant.

3. Results

3.1. Expression of bcl-2 and bax

Figs. 1 and 2 show the effect of Co^{2+} and Cr^{3+} ions on the expression of bcl-2 and bax in U937 human macrophages. Fig. 1 is a representative example of Western blots while the analysis of results is presented in Fig. 2. Our results show that Co^{2+} ions inhibited Bcl-2 expression in a time-dependent manner with significant effect ($p < 0.05$) after 16 h and a maximal 52% inhibitory effect after 24 h with 10 ppm (Fig. 2A). Co^{2+} stimulated Bax expression in a time-dependent manner with significant effect ($p < 0.05$) after 8 h and a maximal 1.75-fold increase after 16 h with 10 ppm (Fig. 2A).

Cr^{3+} ions also inhibited Bcl-2 expression in a time-dependent manner with significant effect ($p<0.05$) after 16 h and a maximal 43% inhibitory effect after 24 h with 500 ppm (Fig. 2B). Cr^{3+} stimulated Bax expression in a time-dependent manner with significant effect ($p<0.01$) after 8 h and a maximal 2.25-fold increase after 24 h with 500 ppm (Fig. 2B).

In parallel, Co^{2+} and Cr^{3+} ions induced a 2.5-fold increase in the expression of PARP fragment, confirming the induction of apoptosis by these ions in U937 macrophages (Fig. 1).

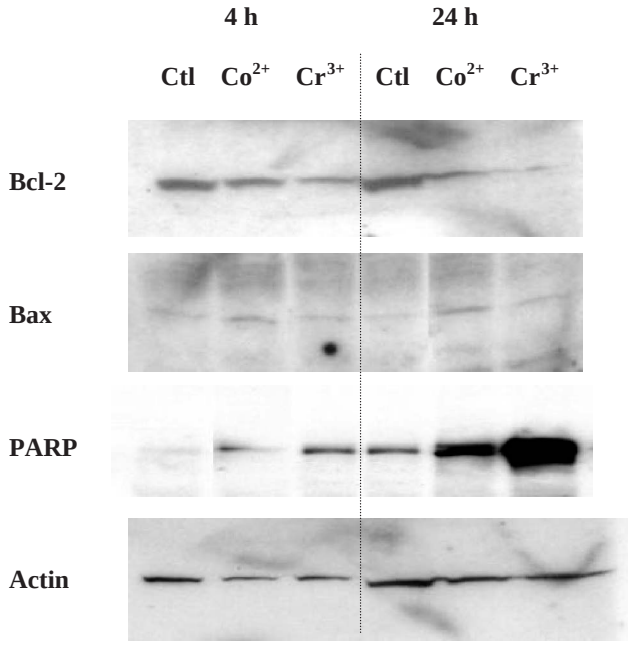

Fig. 1. Western blot analysis of Co^{2+} and Cr^{3+} ions effect on bcl-2 and bax expression. U937 macrophages were incubated for 0–24 h in the presence of 10 ppm Co^{2+} and 500 ppm Cr^{3+}. Protein expression was analyzed by Western blot as described in Section 2. Results are representative of four different experiments.

3.2. Expression of caspase-3 and caspase-8 active fragments

Figs. 3 and 4 show the effect of Co^{2+} and Cr^{3+} ions on the expression of caspase-3 and caspase-8 active fragments in U937 human macrophages. Fig. 3 is a representative example of Western blots while the analysis of results is presented in Fig. 4. Our results show that Co^{2+} ions stimulated the expression of caspase-3 active fragment (17 kDa) in a time-dependent manner with significant effect ($p<0.05$) after 4 h and a maximal 8.0-fold increase after 24 h with 10 ppm (Fig. 4A). In the same experiments, Co^{2+} ions had no effect on the expression of caspase-8 active fragment (18 kDa) (Fig. 4A).

Cr^{3+} ions stimulated the expression of both caspase-3 and -8 active fragments. The stimulation of caspase-3 was time-dependent with significant effect ($p<0.05$) after 8 h and a maximal 4.0 fold increase after 24 h with 500 ppm (Fig. 4B). The effect of Cr^{3+} ions on the expression of caspase-8 was significant ($p<0.05$) after

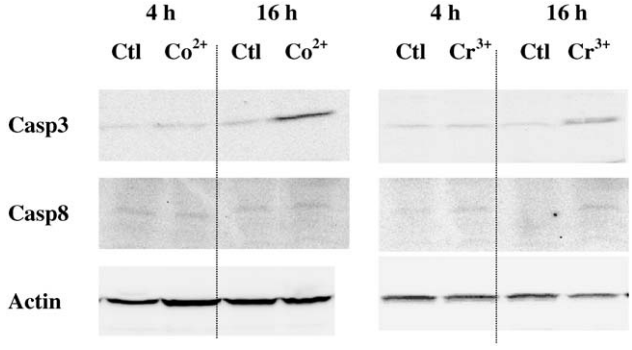

Fig. 3. Western blot analysis of Co^{2+} and Cr^{3+} ions effect on caspase-3 and -8 expression. U937 macrophages were incubated for 0–24 h in the presence of 10 ppm Co^{2+} and 500 ppm Cr^{3+}. Protein expression was analyzed by Western blot as described in Section 2. Results are representative of four different experiments.

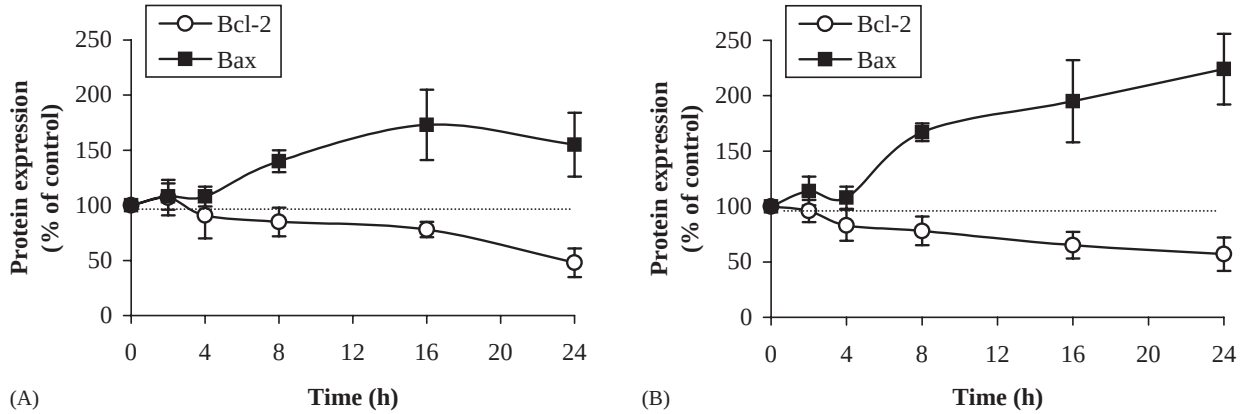

Fig. 2. Effect of Co^{2+} and Cr^{3+} ions on the expression of bcl-2 and bax. U937 macrophages were incubated for 0–24 h in the presence of 10 ppm Co^{2+} (A) and 500 ppm Cr^{3+} (B). Protein expression was analyzed by Western blot as showed in Fig. 1. Results are the mean ± SD of four experiments.

4 h, reached a maximal 3.25-fold increase after 8 h with 500 ppm, and decreased after 24 h (Fig. 4B).

3.3. Activity of caspase-3 and caspase-8

Figs. 5 and 6 show the effect of Co^{2+} and Cr^{3+} ions on caspase-3 and -8 activities in U937 human macrophages. Results show that Co^{2+} ions induced caspase-3 activity with a significant effect ($p<0.05$) after 4 h incubation and a maximal 2.65-fold increase reached after 24 h with 10 ppm (Fig. 5A). This effect was dose-dependent with significant increase ($p<0.05$) with 4 ppm (Fig. 6A). Co^{2+} had no effect on caspase-8 activity (Figs. 5A and 5B).

Cr^{3+} ions significantly stimulated caspase-3 activity after 4 h with a maximal 1.75-fold stimulation after 24 h (Fig. 6A). This was 50% of what was observed with Co^{2+}. This effect was dose-dependent with significant increase ($p<0.05$) with 250 ppm (Fig. 6B). On the other hand, Caspase-8 activity was significantly increased ($p<0.01$) after 2 h incubation. It reached a maximal 2.2-fold increase after 8 h and decreased thereafter (Fig. 6A). Indeed, the dose-dependent analysis of results obtained after 24 h incubation shows a biphasic effect with maximal stimulatory effect reached with 250 ppm Cr^{3+} and a decrease at higher concentrations.

4. Discussion

Using a mouse macrophage cell line, our previous work suggested that Co^{2+} and Cr^{3+} ions induce apoptosis via, at least in part, a caspase-3 pathway [5,6]. In the present study, we used a human cell line of monocyte/macrophage (U937) that is already used for the study of orthopaedic wear particles [15–18] and has comparable responses to polyethylene particles [17] and metal ions [18] to macrophages in primary culture with regards to cytokine release. Results suggest that the induction of macrophage apoptosis by Co^{2+} and Cr^{3+}

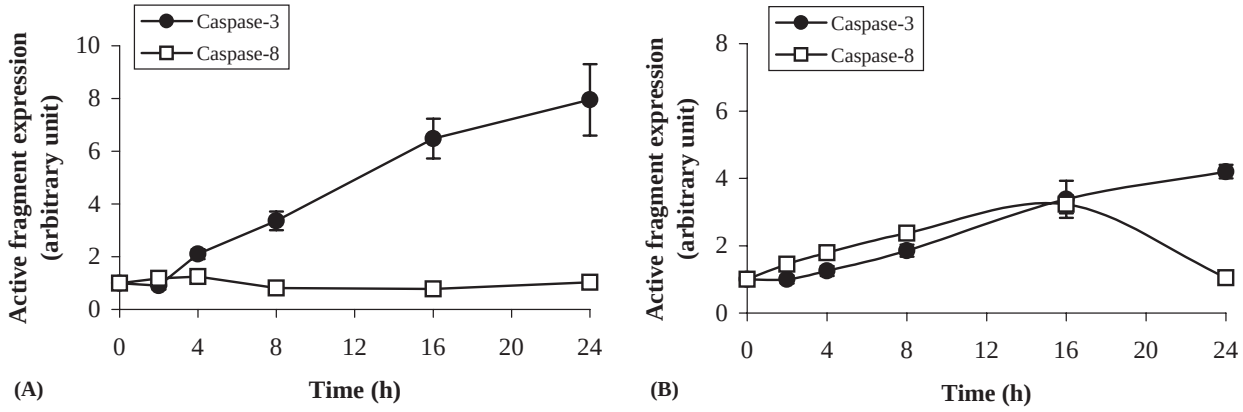

Fig. 4. Effect of Co^{2+} and Cr^{3+} ions on the expression of caspase-3 and -8 active fragments. U937 macrophages were incubated for 0–24 h in the presence of 10 ppm Co^{2+} (A) and 500 ppm Cr^{3+} (B). Protein expression was analyzed by Western blot as showed in Fig. 3. Results are the mean ± SD of four experiments.

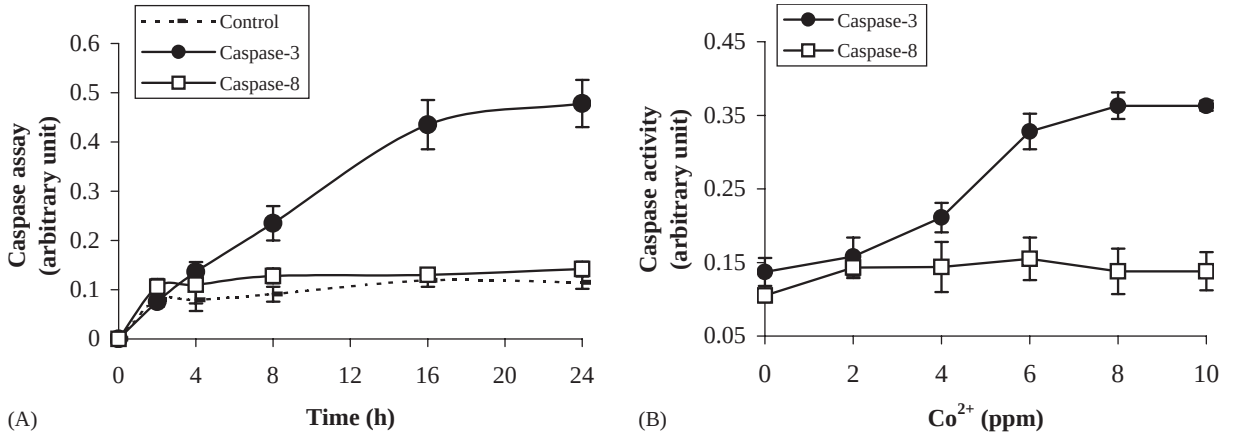

Fig. 5. Effect of Co^{2+} ions on caspase-3 and -8 activities. U937 macrophages were incubated for 0–24 h with 10 ppm Co^{2+} (A) or for 24 h with 0–10 ppm Co^{2+} (B). Caspase activities were measured by colorimetric assays as described in Section 2. Results are the mean ± SD of three experiments performed in duplicate.

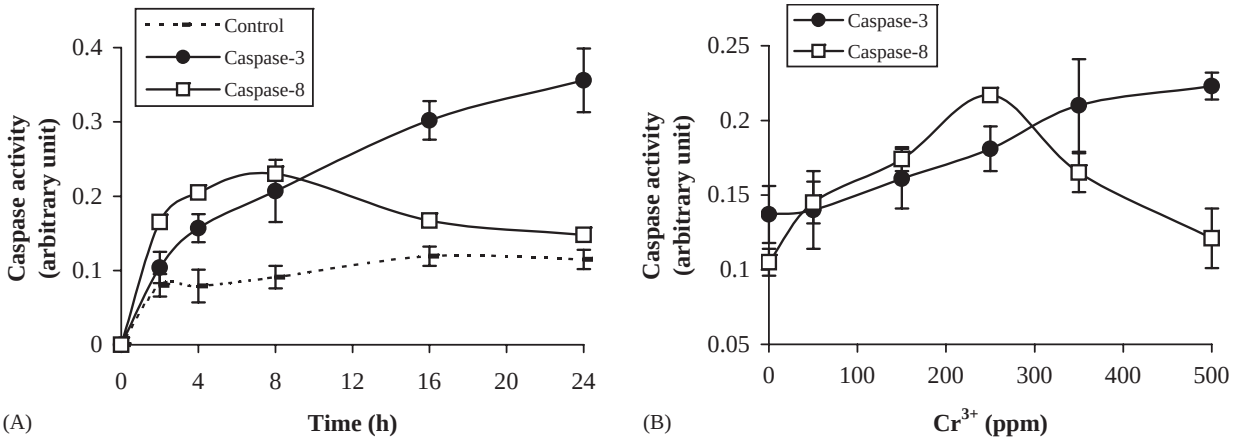

Fig. 6. Effect of Cr^{3+} ions on caspase-3 and -8 activities. U937 macrophages were incubated for 0–24 h with 500 ppm Cr^{3+} (A) or for 24 h with 0–500 ppm Cr^{3+} (B). Caspase activities were measured by colorimetric assays as described in Section 2. Results are the mean ± SD of three experiments performed in duplicate.

ions requires the modulation of the expression and activity of several apoptosis-related proteins.

Our results show that the induction of human macrophage apoptosis by Co^{2+} and Cr^{3+} ions is occurring, at least in part, through the inhibition of bcl-2 and the induction of bax expression. In IMs of aseptically loose THA, the presence of these proteins did not correlate with the presence of apoptosis [9]. These results suggest that the expression of these proteins *per se* does not harm cells under physiological conditions [19]. It was proposed that the ratio between bax and bcl-2 is more important in the regulation of apoptosis than the level of each protein separately [20]. In this regard, we observed significant increases of the bax/bcl-2 ratios with both Co^{2+} (3.4-fold increase) and Cr^{3+} (7.0-fold increase). The later also suggests that the proteins of the bcl-2 family of proteins are more implicated in the macrophage response to Cr^{3+} than for Co^{2+} ions.

It is well known that the proteins of the bcl-2 family play a pivotal role in cells undergoing apoptosis by interfering with the caspases [7,8]. However, the increase (bax) or decrease (bcl-2) of expression of these proteins was slower to what observed for the expression of caspase active fragments that are rapidly activated by proteolysis without protein synthesis as for bax, or protein degradation as for bcl-2. This suggests that modulation of bcl-2 and bax expression by Co^{2+} and Cr^{3+} may be initiated by caspases. We observed differential effect of Co^{2+} and Cr^{3+} ions on the expression of caspase-3 and -8 active fragments. This differential effect was also observed for the activation of caspase activities, confirming the specificity of the caspase substrates used in our experimental model. Indeed, Figs. 4A and 5A, as well as Figs. 4B and 6A, showed similar pattern of activation with variations in time and amplitude of the responses, probably due to a different sensibility of the techniques (Western blot vs. enzymatic assay).

Since caspase-8 is the prototypic initiator of the death domain receptor pathway of apoptosis and is primarily activated by membrane associated events [20], our results suggest that Cr^{3+} ion interacts with cell membrane components to induce macrophage apoptosis. The activation of caspase-8 by Cr^{3+} is transient and probably serves as the initiator of a death pathway that included caspase-3 [21,22]. Results also suggest that other apoptosis-related proteins may also be implicated in the macrophage response to Cr^{3+} ions. Indeed, it was reported that the cleavage of bid, another protein of the bcl-2 family, by caspase-8, is required for death receptor signaling to mitochondria [23]. Another study established caspase-6 as a major activator of caspase-8 in vitro [24]. The role of these proteins in the modulation of macrophage apoptosis by Cr^{3+} ion remains to be investigated.

The dose-response to Cr^{3+} shows an increased caspase-8 activation up to 250 ppm with a decrease thereafter. This correlates with our previous results showing a decreased secretion of TNF-α at these Cr^{3+} concentrations and suggests a highly toxic effect [6]. The macrophage response to Cr^{3+} may imply that the binding of high concentrations of Cr^{3+} to the cell blocks, in a non-specific manner, the membrane enzyme activity (e.g. caspase-8) and membrane traffic (e.g. TNF-α release).

The fact that Co^{2+} ions did not stimulate caspase-8 activity suggests that it might stimulate caspase-3 and apoptosis by a different mechanism to that stimulated by Cr^{3+}, which might be initiated intracellularly. This is in agreement with recent results suggesting that Co^{2+} ions can be transported inside Chinese hamster ovary (CHO) cells transfected with a specific transporter (natural resistance-associated macrophage protein-2; Nramp2) that deliver the ions (Fe^{2+}, Co^{2+}, and Cd^{2+} but not Mg^{2+}) into the cytoplasm [25]. The exact intracellular targets of these ions remain to be identified.

Nevertheless, the implication of caspase-3 in the induction of apoptosis by both Co^{2+} and Cr^{3+} ions is in agreement with previous studies indicating that caspase-3 activation plays a major role when U937 cells undergo apoptosis [26,27].

In conclusion, our results suggest that the modulation of the expression of proteins from the bcl-2 and the caspase families are implicated in the induction of apoptosis by Co^{2+} and Cr^{3+} ions in macrophages in vitro. Moreover, our results suggest that Cr^{3+} ions interact with cytoplasmic membrane components whereas Co^{2+} ions are transported into the cell where they activate intracellular pathways.

Acknowledgements

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