Chromium chloride cytotoxicity and cytokines production in BALB/c cell line

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Abstract

The aim of this study was to examine the cytotoxic effect of the chromium chloride on mouse fibroblasts. The experiments were performed on the BALB/c 3T3 (mouse BALB/c embryo fibroblasts) cell line. The cell viability was measured by three tests: the MTT reduction, LDH release and Neutral Red uptake. The apoptosis was determined by photometric enzyme- immunoassay. Moreover, the IL-1α and IL-6 concentration was determined.

The present study showed that chromium chloride decreases cells viability (confirmed in three cell viability tests) after 24 h of incubation. The suggested mechanism of chromium action in mouse embryo fibroblasts can be presented as follows: the chromium in cells interacts first with mitochondria, then with cell membrane and finally with lysosomes. Moreover, the observed increase of mono- and oligonucleosomes in the cytoplasm of cells is caused by the fact that DNA degeneration occurs several hours before the plasma membrane breakdown. Moreover, chromium chloride increases IL-1α and decreases IL-6 concentration secreted into the cell culture supernatant.

Key words: *chromium, cytotoxicity, apoptosis, cytokines.*

(Centr Eur J Immunol 2010; 35 (2): 58-62)

Introduction

Chromium is an element commonly occurring in nature in trivalent (Cr III) and hexavalent (Cr VI) forms [1]. The reduction of the hexavalent chromium to the trivalent chromium results in formation of reactive intermediates, such as reactive oxygen species (ROS) including superoxide anion, singlet oxygen and hydroxyl radicals, as well as Cr V and Cr IV intermediates, which may interact with microfilaments, mitochondria, lysosomes and nucleus [1, 2]. Inside the cells chromium Cr (III) compounds can directly bind with DNA *in vitro*, forming Cr-DNA adducts and DNA-DNA crosslinks [3].

Nevertheless, the chromium is essential for proper insulin function and is required for normal protein, fat and carbohydrate metabolism [4]. The signs and symptoms of chromium deficiency in mammals are as follows: impaired glucose tolerance, elevated circulating insulin, glycosuria, fasting hyperglycemia, impaired growth, elevated circulating cholesterol and triglyceride concentrations, decreased insulin receptor number and impaired humoral immune response [5]. The effects of chromium supplemented diet have been examined in numerous animal and human studies, but the results of investigations are not equivocal.

Materials and methods

Chemicals and materials

The Dulbecco's Modified Eagle Medium (DMEM), the heat-inactivated Fetal Bovine Serum (FBS), antibiotic/antimycotic (penicillin, streptomycin, amphotericin B), chromium chloride ($[Cr(H_2O)_4Cl_2]Cl \times 2H_2O$), Neutral Red Kit and LDH Kit, were purchased from Sigma Chemical Co., (St. Louis, MO, USA). The MTT Kit, IL-1α and IL-6 ELISA Kits were obtained from R&D Systems Europe (UK). The Cell Death Detection ELISA Kit was obtained from Roche (Germany). Phosphate-buffered saline (PBS) and 0.25% trypsin were purchased from Biomed (Lublin, Poland) and tissue culture dishes were purchased from Nunc Brand Products (Denmark).

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Cell culture and treatment

Mouse embryo fibroblasts (cell line BALB/c 3T3) were obtained from dr D. Śladowski (Department of Transplantology & Central Tissue Bank, Centre of Biostructure, The Medical University of Warsaw). The cells were cultured as adherent monolayers in plastic tissue-culture dishes in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) heat-inactivated Fetal Bovine Serum (FBS) and penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (0.25 µg/ml). Cells were maintained at 37˚C in humidified incubator in atmosphere containing 5% CO_2. The cells were subcultured three times a week treating with 0.25% trypsin at 37˚C for 5 min. Cells were used for cytotoxicological assays during exponential phase of growth.

$[Cr(H_2O)_4Cl_2]Cl \times 2H_2O$ was dissolved in PBS at the concentration of 1 mM. The final concentration was obtained by the dilution in culture medium (DMEM) supplemented with FBS and antibiotics.

In order to perform MTT, LDH, NR assays, apoptosis detection assay, IL-1α and IL-6 assays, the cells were cultured on 96-well plates (2×10^5 cells/ml) in 100 µl DMEM, supplemented with 10% FBS and antibiotics. After 24 h of incubation the medium was exchanged for the fresh DMEM supplemented with $[Cr(H_2O)_4Cl_2]Cl \times 2H_2O$ at final concentrations as follows: 0 (control), 50, 100, 200, 300, 400, 500, 600, 700 and 800 µM for MTT, LDH, NR assays and the apoptosis detection assay. On the other hand 0 (control), 50, 100, 300, 500 and 700 µM $[Cr(H_2O)_4Cl_2]Cl \times 2H_2O$ for IL-1α and IL-6 assays. All concentrations mentioned above are the final concentrations in the incubations. After 24 h of incubation assays were performed according to the original manufacture's instruction. The absorbance in MTT, LDH, NR assays and the apoptosis detection assay was measured in the use of the microplate reader at 620 nm wavelength for MTT assay, 490 nm wavelength for LDH, NR, apoptosis detection assays, and 450 nm wavelength for IL-1α and Il-6 assays. All experiments were performed independently at least six times.

The MTT assay: the IC_{50} (concentration causing 50% reduction in growth compared with the control) value was calculated from the graph of the dose-response curve.

The apoptosis analysis: After the experiment the enrichment factor of mono- and oligonucleosomes released into the cytoplasm was calculated:
n (enrichment factor) = the absorbance of the sample/the absorbance of corresponding negative control.

Interleukin 1α and interleukin 6 cytokine measurement

Cytokines (IL-1α and IL-6) concentrations were measured by the sandwich-linked immunosorbent assay with the use of commercially available kits (R&D Systems) according to the manufacture's instruction. A standard curve was constructed by plotting the absorbance of each standard vs. the corresponding standard concentration and then, the cytokine levels of unknown samples were calculated. The sensitivities of assays were as follows: 2.5 pg/ml for IL-1α and 1.6 pg/ml for IL-6.

Statistical analysis

Results from MTT, LDH, NR, apoptosis and cytokines assays were analysed with the use of Student's t-test with computer assistance (Statistica program). The accepted level of significance in all cases was $p < 0.05$. All results are presented as mean values ± SD

Results

The results of the cell viability after incubation with chromium chloride at various concentrations are displayed on Fig. 1-3. It can be seen from the Fig. 1 that there was a concentration-dependent decrease in the cell viability

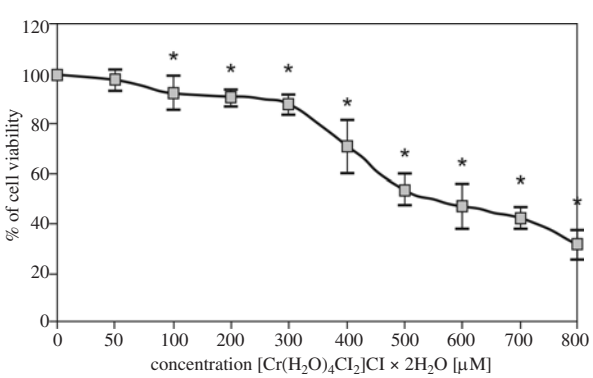

Fig. 1. Cytotoxic effect of cromium chloride in BALB/c 3T3 line detected with the MTT reduction assay. Values are given as percentage of cell viability relative to cells without chromium (control); *$p < 0.05$, significance of difference copmared with control

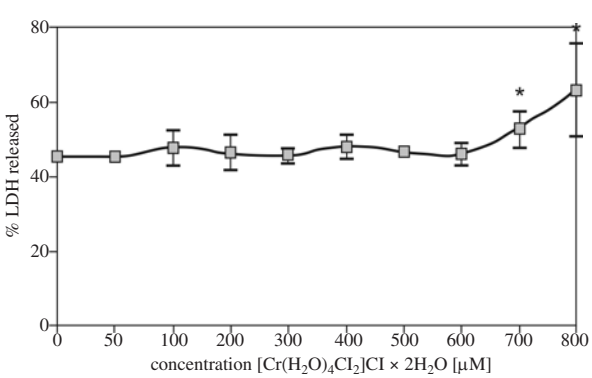

Fig. 2. Cytotoxic effect of cromium chloride in BALB/c 3T3 line detected with the LDH release assay. Values are given as percentage of LDH relese relative to cells without chromium (control); *$p < 0.05$, significance of difference copmared with control

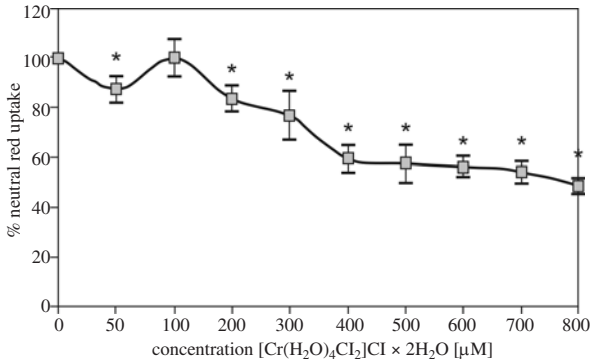

Fig. 3. Cytotoxic effect of cromium chloride in BALB/c 3T3 line detected with the neutral red uptake assay. Values are given as percentage of neutral red uptake relative to cells without chromium (control); *$p < 0.05$, significance of difference copmared with control

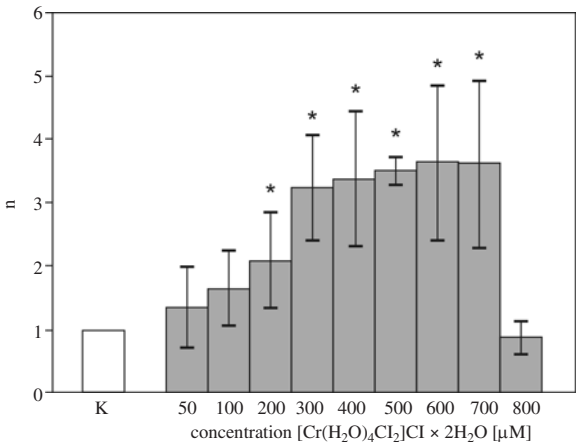

Fig. 4. Apoptosis in 3T3 cells induced by chromium chloride; *$p < 0.05$, significance of difference copmared with control

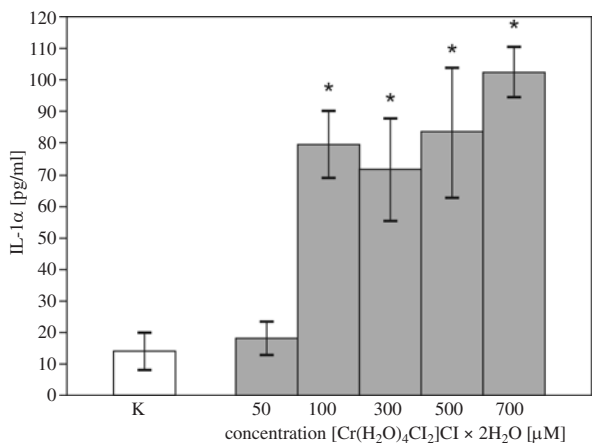

Fig. 5. The interleukin IL-1α concentrations; *$p < 0.05$, significance of difference copmared with control

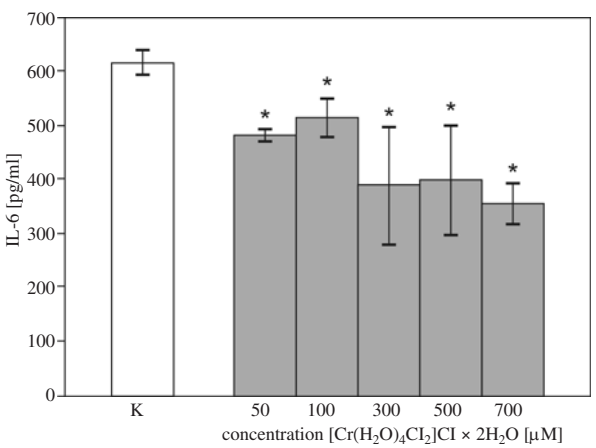

Fig. 6. The interleukin IL-6 concentrations; *$p < 0.05$, significance of difference copmared with control

(statistical significantly). Chromium chloride was toxic above the concentration of 100, 200, 300, 400, 500, 600, 700, 800 μM (Cr III reduced cell viability to 92, 90, 87, 70, 53, 46, 42 and 31%, respectively).

The cell viability was also determined by LDH release assay (Fig. 2). BALB/c 3T3 fibroblasts exposed to chromium chloride demonstrated a concentration-dependent increase of LDH release form the cells. Chromium chloride was toxic above the concentration of 700, 800 μM.

Cell viability was also determined by Neutral Red uptake assay. It can be seen from the Fig. 3 that there was a concentration-dependent decrease (statistically significant) in Neutral Red uptake.

Figure 4 demonstrates concentration-dependent increase of n factor. Apoptosis increases significantly at all concentrations apart form concentration of 800 μM.

Figure 5 shows IL-1α levels induced by chromium chloride. It can be seen that chromium increase statistically significant IL-1α concentration after incubation with 100, 300, 500, 700 μM of chromium chloride, whereas chromium chloride decreases statistically significant IL-6 concentration (Fig. 6).

Discussion

The aim of presented work was to investigate the effect of chromium (III) on fibroblasts. The BALB/c 3T3 cell line was chosen for our investigation as it has been proposed as a cellular model in studying the morphological and biochemical changes induced by biometals [6]. Moreover this cell line is recommended for cytotoxicity tests by European Union (Council Directive 86/609/EEC).

Cytotoxicity was assessed by with the use of three functional assays: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction, the Lactate dehydrogenase (LDH) release and Neutral Red (NR) uptake.

The MTT assay is often used to determine cytotoxicity of the following exposure to toxic substances. This test monitors reduction of the soluble yellow MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to insoluble purple formazan in a reaction catalysed by mitochondrial dehydrogenases in viable cells. As a result, the MTT assay is a marker of mitochondrial function.

Cytotoxicity induced by chromium was also assessed by the LDH assay. The lactate dehydrogenase assay is a way of measuring membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. The assay is based on the reduction of NAD by the action of LDH. The result of reduced NAD (NADH) is utilized in the stoichiometric conversion of a tetrazolium dye. The assay measures LDH-mediated conversion of tetrazolium salt to red formazan products.

The Neutral Red assay is also used to measure the cell viability. Viable cells take up the dye (Neutral Red) by active transport and incorporate the dye into lysosomes, whereas non-viable cells do not take up the dye. After having been allowed incorporate the dye, the cells are briefly washed. The incorporated dye is then liberated from the cells.

The apoptosis was determined by the photometric enzyme-immunoassay. The assay is based on a quantitative sandwich-enzyme-immunoassay- principle with the use of mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmatic fraction of cell lysates. Moreover, the influence on IL-1α and IL-6 production was determined by ELISA assays.This work is a first report discussing the mechanism of chromium action in cells. The suggested mechanism of chromium action in mouse embryo fibroblatst can be presented as follows: chromium in cells interact first with mitochondria, then with cell membrane and finally with lysosomes.

The present study has shown that chromium chloride decreases cells viability (confirmed in three cell viability tests) after 24 h of the incubation. Similar results were obtained by Shrivastava *et al*. using MTT reduction test. They have shown a decrease in the formation of formazan from MTT, which is an indirect indication of mitochondraial activity [1]. Moreover, Rudolf *et al.* have shown that chronic exposure to chromium acetate causes degenerative changes in Hep-2 cells via damaged cell membranes and the induction of apoptosis [2]. That corresponds with our investigations, which have shown that chromium chloride induces apoptosis in fibroblasts.

The observed increase of mono- and oligonucleosomes in the cytoplasm of cells is caused by the fact that DNA degeneration occurs several hours before the plasma membrane breakdown. Catelas *et al*. results obtained with the use of mouse macrophages (J774 cell line) are in agreement with finding reported in our investigations. Chromium chloride caused DNA and histones increase of concentrations in the cell culture supernatant after 24 and 48 h of the incubation [7]. Moreover, the DNA damage was observed in Seoane *et al*. investigations. They have found that incubation of human diploid fibroblasts (MRC-5 cell line) with chromium chloride caused an increase in micronuclei formation [8]. That is confirmed by investigations performed by Błasiak *et al*., which have shown the decrease of the cell viability and the increase of comet tail moments after chromium chloride incubation [9].

Mazzotti *et al*. have shown that chromium chloride in a range of concentrations of 0.1-1000 μM, was not cytotoxic to fibroblasts according to colony-forming efficiency (CFE) test [6]. Similar results have been obtained by Stearns *et al*. on Chinese hamster ovary (CHO) cells. These authors showed that chromium chloride treatment (48 h) did not decrease the cell viability up to 1 mM $CrCl_3$ [10].

It is known that Cr (III) *in vitro* and *in vivo* induce DNA strand breaks, DNA-protein cross links and formation of 8-hydroxydeoxyguanosine. This may result in the apoptosis induction in cells. Manygoats *et al*. demonstrate that chromium chloride-exposed Chinese hamster ovary (CHO AA8) cells have not shown changes in the relative number of normal and apoptotic cells until 1 mM dose [11].

The effect of chromium on cytokines has been studied either by administration of chromium or implantation of prosthesis containing chromium alloy. Investigations performed by Horowitz *et al*. have shown that incubation of macrophages with chromium lead to the release of TNF-α and PGE2, but not IL-1α and IL-6 [12]. Other authors have found a higher release of IL-6 and IL-1α from J744 cell line, lymphocytes, monocytes and human synoviocytes [13-15]. Our investigations have shown the increase of IL-1α concentration and decrease of IL-6 concentration after incubation of mouse fibroblasts with chromium chloride. These discrepancies may be explained by differences in cell types and experimental procedures. Thus, these problems still demand a lot of investigation.

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