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Autoschizis of T-cells is induced by the nutritional supplement, Cr(III)picolinate

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ABSTRACT

In recent times, Cr(III)(picolinate)$_3$ [Cr(III)(pic)$_3$] a nutritional supplement, is gaining attention because of its clastogenic and mutagenic properties. Earlier studies of ours indicated that Cr(III)(pic)$_3$ is cytotoxic to lymphocytes with ROS and mitochondrial events playing a role in bringing about apoptosis. Now, we report that, autoschizis is induced in lymphocytes in a concentration and time dependent manner which is confirmed through TEM and SEM. Lymphocytes treated with concentrations of 100 μM of Cr(III)(pic)$_3$ exhibit features such as cytoplasmic bleb, self excision of cytoplasm, cytoplasmic leakage and membrane bound bodies formed from the excised pieces apart from apoptosis and necrosis. Though autoschizis has been described in tumor cell lines treated with menadione and ascorbate, occurrence of this cell death in normal T-lymphocytes is reported here. The cellular events that accompany autoschizis are found to be increase in intracellular Reactive Oxygen Species (ROS) and cytoplasmic lactate dehydrogenase, loss of mitochondrial membrane potential (MMP) and depletion of ATP. Further, autoschizis is effected through increases in DNase I and DNase II activity with a concomitant decrease in caspase-3 activity which leads to a random cleavage of the DNA as demonstrated by a smear like pattern after electrophoresis on agarose gel.

1. Introduction

Chromium picolinate [Cr(III)(pic)$_3$] is widely used as a nutritional supplement due to its beneficial role in reducing serum cholesterol and glucose levels. This is a well absorbed form of chromium compared to dietary chromium that has an uptake efficiency of less than 2% (Anderson et al., 1997; Vincent, 2001, 2003). However, questions regarding the safety of Cr(III)(pic)$_3$ have been raised due to its reported mutagenic and clastogenic properties (Stearns et al., 1995; Stearns and Silveira, 2002). Renal failure, liver dysfunction, contact dermatitis and destruction of skeletal muscles have been observed when 600 μg of Cr(III)(pic)$_3$/day for 6 weeks are administered to humans (Wasser et al., 1997). Evidence for oxidative damage and DNA fragmentation in cultured J77A.1 macrophage cells, chromosomal aberrations in *Drosophila melanogaster* have been reported (Bagchi and Stohs, 2002; Bagchi and Bagchi, 1997; Stallings et al., 2006). Lay and coworkers have suggested that the products of these ligand exchange reactions may increase its susceptibility to oxidants such as H_2O_2 and glucose oxidase system leading to the formation of Cr(IV), Cr(V) and Cr(VI). While Cr(IV) and Cr(V) are reactive radicals which can damage macromolecules, Cr(VI) reduction can yield Cr(V), Cr(IV) and Cr(III) all of which can contribute to the biotoxicity of Cr(III)(pic)$_3$ (Levina and Lay, 2008). Apoptosis of CHO cells has been reported when treated with Cr(III)(pic)$_3$ (Manygoats et al., 2002). Intravenous injection of ^{51}Cr(^{3}Hpic) daily for 14 days to rats has indicated that both the labels are distributed in various tissues but predominantly found in the liver (Hepburn and Vincent, 2002). Further, it has been demonstrated that Cr(III)(pic)$_3$ resides intact in the blood for 30 min and then enters the liver and kidney (Hepburn and Vincent, 2003). Correlations between increased amount of 8-hydroxydeoxyguanosine and lipid peroxidation in liver and kidney tissues with that of urinary levels have been made (Hepburn et al., 2003a,b). A very recent *in vivo* study has reported cytotoxicity, increased levels of lipid peroxidation and micronuclei formation in the lymphocytes of calves which received low concentrations (200 μg and 400 μg) of chromium as Cr(III)(pic)$_3$ for 12 weeks via oral administration (Imamoglu et al., 2008). On the other hand, rats fed up to 100 mg of Cr as Cr(III)(pic)$_3$/kg for 24 weeks did not exhibit any toxic response (Anderson et al., 1997). Thus there are contradictory observations and thereby, a need for further investigations on the effects of Cr(III)(pic)$_3$.

Various cell death forms have been identified which are physiological, pathological or those resulting from toxic insults in different cells and cell lines. Apoptosis and necrosis are the two distinct forms of cell death identified based on morphological and biochemical characteristics. Apoptosis is characterized by cell shrinkage, chromatin condensation, membrane bleb formation and fragmentation of membrane bound apoptotic bodies whereas

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necrosis is characterized by swelling of the cell, disruption of cell membrane and the release of cell contents into the extra cellular space (Kerr et al., 1972; Wyllie et al., 1980; Manjo and Joris, 1995; Georg Häcker, 2000; Fesus, 1993; Proskutyakov et al., 2003). Different stages of these forms which combine the features of both apoptosis and necrosis have also been demonstrated. One modified form of cell death which has characteristic features different from that of apoptosis and necrosis was first observed in prostate cancer cell lines exposed to a combination of vitamin C (ascorbate) and vitamin K_3 (menadione) by Gilloteaux et al. (1995). Later, in human prostatic carcinoma cell lines treated with the same vitamins, similar features were observed by this group and they coined the term "Autoschizis" for this form of cell death (Gilloteaux et al., 1998; Jamison et al., 1996; Venugopal et al., 1996). Some of the characteristics of autoschizis include cytoplasmic leakage, self excision of cytoplasm, excised pieces forming membrane bound bodies and aggregation of organelles. Human ovarian and bladder carcinoma cell lines, murine prostate cancer cells, and renal carcinoma cells treated with vitamin C and vitamin K_3 also display characteristics of autoschizis (Gilloteaux et al., 1998, 1999, 2003a,b, 2004; Arnold et al., 1999; Jamison et al., 2002). Earlier in our laboratory, we have shown that, chromium (III) complexes containing aromatic ligands -tris(2,2′-bipyridyl)chromium(III)chloride ($[Cr(bpy)_3]Cl_3$), tris(1,10-phenanthroline) chromium(III) chloride ($[Cr(phen)_3]Cl_3$) induce apoptosis of lymphocytes (Rajaram et al., 1995; Balamurugan et al., 2002, 2004). Very recently, we have demonstrated that, concentrations of 25 and 50 μM $Cr(III)(pic)_3$ induce apoptosis of human peripheral lymphocytes and that ROS are the precursor molecules in this apoptotic process which further leads to loss of MMP and activation of caspase-3 (Jana et al., 2009). When we increase the concentration to 100 μM and above, the level of caspase-3 in lymphocytes seems to reduce indicating that apoptosis may be blocked. It is not known whether these cells are diverted to undergo some other mode of cell death. In order to find out if this is so, lymphocytes have been treated with concentrations of $Cr(III)(pic)_3$ at which caspase-3 activity is reduced and the morphology by optical and electron microscopy as well as some biochemical parameters have been evaluated. Apart from apoptosis, we report that a differed form of cell death is observed in peripheral blood lymphocytes when treated with concentrations of 100 μM and above of $Cr(III)(pic)_3$.

2. Materials and methods

2.1. Reagents

RPMI 1640 medium, Hank's balanced salts solution (HBSS), Ficoll-Hypaque-1077, heparin, penicillin–streptomycin, gentamycin, amphotericin-B, phythaemagglutinin (PHA), ribonuclease A, SDS, Triton X-100, NP-40, agarose, proteinase K, 3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) May-Grünwald Giemsa, ethidium bromide (EtBr), acridine orange (AO), 4′-6-Diamidino-2-phenylindole (DAPI), 2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), NADH, pyruvic acid, H_2O_2, sodium pyruvate, hexamethyldisilazane (HMS), herring sperm DNA, sodium acetate, DNase I (EC 3.1.21.1), DNase II (EC 3.1.22.1), picolinic acid (PA) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were purchased form Sigma, USA. CD-3 micro bead was purchased from MACS, Miltenyi Biotech, Germany. Cell Titer-glo^R Luminescent kit was purchased from Promega, USA. N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was purchased from BD Biosciences, USA. [^{3}H]-thymidine was sourced from Board of Radiation and Isotope Technology (Mumbai, India). Sodium cacodylate, glutaraldehyde, osmium tetroxide, uranyl acetate, lead citrate, Spurr's resin-kit were purchased from EM Sciences, USA. Chromium picolinate [$Cr(III)(pic)_3$] was prepared and characterized according to the methods described earlier (Evans and Pouchmik, 1993). All other reagents were of pure or analytical grade and buffers were filtered through 0.45 μm Millipore filters.

2.2. Isolation of T-cells

The peripheral blood mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation as described (Boyum, 1968). The buffy coat containing the mononuclear cells was aspirated, washed twice with Mg^{2+} and Ca^{2+} free PBS. Monocytes were removed by adherence to plastic for 45 min. T-cells were separated using magnetically labeled CD-3 micro beads (MACS, Miltenyi Biotech, Germany) as per manufacturer's protocol. The T-cells were collected and cultured in RPMI 1640 medium containing 2 mM glutamine, 10% autologous plasma, 100 IU/mL each of penicillin and streptomycin and maintained at 37 °C and 5% CO_2 in a CO_2 incubator (Binder, Germany). Cells were found to be viable to the extent of 95% during different batches of preparation, as verified by trypan blue dye exclusion method. Cells were treated with different concentrations of $Cr(III)(pic)_3$ or PA for various time periods.

2.3. Cytotoxicity studies

2.3.1. MTT assay

Cell viability was assessed using MTT assay (Campling et al., 1991). For this, cells (2.5×10^4) were treated with different concentrations of $Cr(III)(pic)_3$ or PA for various time intervals and were incubated with MTT (5 mg/mL) in RPMI 1640 medium for 4 h at 37 °C. The medium was removed by centrifugation and the formed formazan product was solubilized with acidified isopropyl alcohol containing 0.05 M hydrochloric acid. Absorbance at 570 and 660 nm were measured for assessing cell survival and background respectively using Specord-200 UV-spectrometer (Analytik Jena, Germany). The percentage of viable cells was calculated with reference to control.

2.3.2. DNA synthesis by [^{3}H]-thymidine incorporation

PHA (12.5 μg/mL) induced proliferation of lymphocytes was measured using [^{3}H]-thymidine incorporation assay (Lee, 1974). Lymphocytes (0.2×10^6/mL) were exposed to 100 to 300 μM of $Cr(III)(pic)_3$ or PA for 24, 48 and 72 h and pulsed with [^{3}H]-thymidine (10 μCi/well). The cells were harvested onto glass filter fibers, using Combi cell harvester (Skatron, Inc., VA 20166, USA) and the amount of [^{3}H]-thymidine incorporated into the cells was quantified using a liquid scintillation counter (Wallac 1409, Sweden).

2.3.3. Analysis and evaluation of cell death by microscopy

2.3.3.1. Giemsa staining. Cells treated with $Cr(III)(pic)_3$ or PA were smeared on glass slides, fixed with dehydrated methanol, stained with May-Grünwald Giemsa stain, washed, dried and visualized under an optical microscope (Nikon Eclipse E600, Japan). About 200 cells were counted and three batches of experiments were carried out. Cells exhibiting condensed chromatin or apoptotic bodies were counted as apoptotic cells and other features of cell death were also noted. Comparison of cells treated with $Cr(III)(pic)_3$ or PA were made against untreated cells.

2.3.3.2. DAPI (4′-6-diamidino-2-phenylindole) staining. Treated cells were permeabilized with 0.1% Triton X-100 and then incubated with fluorescent dye DAPI (1 μg/mL) in methanol for 30 min in the dark. The cells were washed with PBS and then observed using a fluorescence microscope (Ex: 345 nm; Em: 455 nm) (Leica, TCS SP 2-X1, Germany).