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APPROACHES TO A MOLECULAR INSIGHT INTO CHROMIUM(III) INDUCED APOPTOSIS OF HUMAN LYMPHOCYTES

THIRUMALACHARI RAMASAMI, RAMA RAJARAM, KUPPUSAMY BALAMURUGAN, CHELLAPPA VASANT AND KUMARI MURTHY ANANTH

Central Leather Research Institute, Adyar, Chennai-600 020 (India)

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Programmed death of cells, Apoptosis, in human lymphocytes has been observed during *in vitro* proliferation of cells in the presence of some select Schiff base and polypyridyl complexes of chromium(III). A mechanistic and molecular insight into the apoptotic processes induced by chromium(III) complexes is presented. Direct experimental evidence for chromium(III) induced up regulation of Src-family tyrosine kinases as well as changes in caspase (cysteine rich aspartate dependent proteases) promoted pathways is presented. Some chromium(III) complexes seem to alter signaling pathways in cell biology by promoting the formation of Reactive Oxygen Species (ROS). A molecular basis for the chromium(III) induced apoptosis in human lymphocytes has now been proposed.

Key Words : Chromium (VI/V/III); Carcinogenicity; Disproportionation; Reactive Oxygen Species; Src-family Tyrosine Kinases; Caspase-3; Apoptosis; Free Radicals; Phosphorylation

Introduction

Whereas many chromium salts have found wide industrial applications, their toxicological consequences to biosystems have attracted attention[1-4]. There are earlier reports that chromium(VI) salts are carcinogenic, mutagenic and cytotoxic[5,6]. Generally transport of chromium(III) salts into the cellular systems is considered difficult[7,8]. This has led to an assignment of lower biotoxicity potential to chromium(III) than those for chromium(VI) systems[9,10]. Programmed cell death induced by chromium(III) salts was first recognized in the case of two Schiff base complexes *viz. trans*-diaquaethylene-bis[salicylideniminato]chromium(III) perchlorate and its higher homologue[11].

Whereas two specific Schiff base complexes of chromium(III) induced apoptosis in human lymphocytes, many other simpler complexes did not elicit such effects. Among the various complexes investigated in the previous study[11], the two Schiff base complexes were known to undergo oxidation to afford relatively stable Cr(V) products[12,13]. Accessibility of chromium(V) state was considered as possibly one of the important factors associated with the biotoxicity of chromium[11,14]. It is generally believed that Cr(V) compounds disproportionate readily into chromium(VI) and (III) forms[15]. However, a limited number of Cr(V) complexes have been reported to be relatively stable under special conditions[16]. Potentials for the intermediate formation of chromium(V) complexes of bio-macromolecules were investigated through a model study involving chromate ions and bovine serum albumin (BSA)[17]. It was possible to monitor the formation of Cr(V) intermediates using EPR techniques. A characteristic EPR signal attributable to the formation of a relatively stable chromium(V) (for period up to 12 hrs) was observed during the reaction of chromium(VI) with BSA[17]. These observations raise questions as to whether biotoxicity of chromium is coupled to any other redox pathways in cell biology. Redox processes in chemistry of simple chromium compounds are known to be influenced significantly by the nature of ligand environments around the metal ion[18].

The need for investigations of the speciation aspects of biotoxicity of chromium(III) has been recognized[11]. Increasing clinical use of chromium(III)-picolinate and other complexes is made in the control of diabetes[19]. In light of these clinical practices, a molecular understanding and insight into biotoxicity aspects of complexes of the trivalent metal ion and ensuring of the bio-safety of the use of chromium(III) in control of diabetes are needed. Since apoptosis represents a programmed cell death, any mechanistic understanding is required to trace the role of chromium in signalling pathways involved in cell biology. Factors such as

depletion of adenosine triphosphate (ATP), changes in the expression and regulation of tyrosine family of kinases as well as caspase related pathways are known to play vital roles in cellular apoptosis[20-22].

An attempt has now been made to study the role of chromium(III) in signalling pathways in human lymphocytes. Chromium(III) induced non-enzymatic phosphorylation of proteins through group transfer mechanisms has been reported[23]. The present investigation provides evidence that chromium(III) induced apoptosis is related to the formation of reactive oxygen species, which in turn up-regulate Src-family of tyrosine kinases and consequently alter caspase-dependent pathways in cell biology. An effort has been made to propose a molecular approach to the understanding of chromium(III) induced apoptosis in human lymphocytes and the resulting biotoxicity.

Experimental

Materials

Chemicals employed were of reagent grade and were used as procured from supply houses. Biochemicals needed were supplied by Sigma, Calbiochem and R&D systems. Antibodies were supplied by Santa Cruz Biotechnology (CA, USA).

Preparation of Complexes of Chromium

All the complexes of chromium(III) selected for investigations and their preparation procedures were known previously. Reported procedures for preparation and characterization of the chromium(III) complexes needed for this investigation were employed[24-26].

Cr(III)-induced Phosphorylation of Bovine Serum Albumin(BSA)

BSA (3 mM) was treated with 200 mM of Cr(III) complexes and 50 mM of [g-^{32}P]ATP (specific activity 3000 counts per minute (cpm)/pmol) in a volume of 50 mL and incubated at room temperature for 18 h. The reaction was terminated by addition of 12 mL of sample buffer. The samples were heated for 2 min in a boiling water bath and subjected to SDS-PAGE. After staining with Coomassie Brilliant Blue (CBB), the gels were dried and radioactivity determined after auto-radiography. For this, the dried gels were exposed to Konica X-ray film for 2 h at -70° C and then developed. The radioactivity incorporated into the 66 kDa band of BSA was quantitatively estimated using liquid scintillation counting (Wallac 1410, USA). Amount of chromium bound in BSA was estimated using atomic absorption techniques after dialyzing out free chromium. ATP samples labelled at both alpha and gamma centres were employed to distinguish the nature of group transfer namely ATP as a molecule or phosphate as a group. The chromium(III) complexes employed for the study were tris-phenonthrolinechromium(III) chloride, **1**, tris-bipyridylchromium(III) chloride, **2**, trans-diaqua ethylene-bis[salicylaldiminato]chromium(III) perchlorate, **3**, trans-diaqua propylene bis[salicylaldiminato]chromium(III) perchlorate, **4** and Aqua pentamminechromium(III) nitrate, **5**. Phosphorylation of BSA induced by hydroxopentamminechromium(III), **6** was also investigated in the presence of 250 mM of sodium orthovanadate or ammonium molybdate or sodium tungstate using 50 mM of [a-^{32}P]ATP and the extent of phosphorylation estimated.

Chromium Induced Changes in DNA Synthesis and Fragmentation

To 0.75´ 10^6 cells, 10 mg/mL phythemagglutinin (PHA) was added and proliferation of lymphocyte cells in the presence of Cr(III) complexes was analyzed. At selected time intervals, 1 mCi [^{3}H]-Thymidine/well was added and cells harvested at the end of 18 h using Combi cell harvester (Skatron Instruments). The amount of radioactivity was quantitatively estimated using a liquid scintillation counter (Wallac 1409, USA). The percentage inhibition of DNA synthesis upon Cr(III) treatment was then calculated from the radioactivity incorporated into untreated cell. DNA fragmentation was analyzed using agarose gel electrophoresis employing standard and published methods[27].

Quantification of Chromium(III) Induced Changes in DNA-Protein Cross-links (DPC)

DNA fragments containing covalently attached proteins were selectively precipitated in the presence of KCl/SDS and DNA in the cells was analyzed using standard and published procedures and methods[28]. Employing the same procedures, amounts of DNA synthesized in both chromium(III) treated and control cells were estimated. DPC coefficient was estimated from the ratio of amounts of DNA synthesized in the treated and control cells. Effects of antioxidants on the formation of DPC were investigated by pre treating lymphocytes with anti oxidants (*viz.* ascorbic acid (1 mM), a-tocopherol (50 µM), GSH (1 mM),

SOD (500 units/mL), catalase (1000 units/mL) and sodium formate (1 mM) for 18 h.

Morphological Analysis of Chromium Pretreated Human Lymphocyte Cells

Cells treated with Cr(III) complexes were stained with May-Grünwald Giemsa stain and analyzed using a light microscope.

Flow Cytometric Analysis

For analysis of DNA content, cells after treatment with Cr(III) complexes for 48 h were harvested and washed in phosphate-buffered saline (PBS). Cells were fixed in 70% ice-cold ethanol overnight at 4°C. Cells were then stained with a fluorochrome solution (containing 20 mg/mL PI in PBS, 50 mg/mL RNase A and 0.1% Triton X-100) for 30 min at 37°C and analyzed using FACSort flow cytometer equipped with Cell Quest Software (Becton Dickinson, USA).

Quantification of Generation of ROS

Cells (1´ 10^6) after stimulation with Cr(III) complexes for different time periods were suspended in PBS supplemented with 20 mM glucose and incubated with 10 mM dichlorodihydrofluorescein diaceteate (DCFH-DA) at 37°C for 1 h. The fluorescence increase, due to the hydrolysis of DCFH-DA to dichlorodihydrofluorescein (DCFH) by some non-specific cellular esterases and its subsequent oxidation by peroxides was measured.

Effect of Antioxidants on Cr(III) Induced Apoptosis

Lymphocytes were pretreated with ascorbic acid (1mM), GSH (1 mM), a -tocopherol (50 mM), SOD (500 units/mL) and catalase (1000 units/mL) for 18 h. Cells were then exposed to **1** and **4** for designated periods and analyzed for [^{3}H]-Thymidine incorporation, DNA fragmentation and hypodiploid DNA content according to methods described earlier.

Western Blot Analysis

Peripheral blood lymphocyte cells after treatment with Cr(III) complexes **1**, **4** and **5** were harvested at the end of 0.5, 3 and 6 h. Cells were washed in PBS and lysed in a buffer containing Tris 20 mM, NaCl 150 mM, sodium orthovanadate 100 mM, PMSF 100 mM, 10 mg/mL leupeptin, EDTA 500 mM and 0.5% NP-40 for 30 min at 4°C. Cell lysate was clarified by centrifugation at 14,000 rpm for 15 min at 4°C. Protein concentration was determined by Bradford assay[29]. Whole cell lysate (50 mg) was then loaded onto 9% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), electrically transferred onto nitrocellulose membrane and probed with primary mouse monoclonal antibody specific for phosphotyrosine (1:500 dilution). Blots were then washed in Tris-buffered saline containing 0.05% Tween 20, (TBST) incubated with monoclonal anti-mouse IgG-HRP (1:1000 dilution) for 1 h and visualized using DAB as substrate. For Src-family tyrosine kinases cell lysate was immunoprecipitated with anti-phosphotyrosine antibody for 1 h at 4°C. The immune complexes containing phosphoproteins have been collected with protein A/G-Sepharose for 1 h at 4°C, loaded onto 9% SDS-PAGE, electrically transferred onto nitrocellulose membrane and probed with primary rabbit polyclonal antibodies (1:500 dilution) specific for p56lck, p59fyn and p53/56lyn for 16 h at room temperature. Blots were then washed in TBST, incubated with polyclonal anti-rabbit IgG-HRP (1:1000 dilution) for 1 h and visualized as described earlier[30]. Cells were also pretreated with ascorbic acid (1mM), GSH (1 mM), a -tocopherol (50 mM), SOD (500 units/mL) and catalase (1000 units/ mL) for 18 h and then exposed to 25 mM of **1** and **4** for 6 h. Immunoblotting for Src-family tyrosine kinases was carried out as described above.

Immune Complex Kinase Assay

Stimulation of lymphocytes with **1** and **4** and lysis was carried out as described earlier and lck, fyn and lyn were precipitated with specific antibodies (1:100). Immune complexes were collected with Protein A/G-sepharose after three washes with lysis buffer and then washed twice with kinase buffer (20 mM Tris pH 7.0, 10 mM $MnCl_2$, 10 mM $MgCl_2$). Kinase assays were performed by adding 10 mCi of [g-^{32}P]ATP in the presence of 5 mg of acid-treated enolase. After 20 min at 27°C, 5X Laemelli sample buffer was added and the mixture boiled to quench the reaction. The reaction mixture was then run on 9% SDS-PAGE and phosphoprotein analyzed using autoradiography. Kinase activity was quantified from the ^{32}P label incorporated into enolase bands using liquid scintillation counting.

Effect of Src-family Kinase Inhibitor, 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo(3,4-d) Pyrimidine (PP2) on Cr(III) Induced Apoptosis

Cells were pretreated with PP2 (10 mM) for 18 h before addition of **1**and **4.** [^{3}H]-Thymidine incorporation, flow cytometry and DNA fragmentation investigations and Western blotting of cell lysates were carried out as described earlier.

Immunoblotting for Caspase-3

Whole cell lysates were prepared from lymphocytes after being challenged with 25 mM of **1** and **4** for 6, 24 and 48 h. Cells lysate was prepared as described earlier. Whole cell lysate (50 mg) was then electrophoresed on 9% SDS-PAGE, electrically transferred onto nitrocellulose membrane and probed with primary rabbit polyclonal antibody specific for caspase-3 (1:500 dilution). Blots were then washed in TBST (tris-buffered saline containing 0.05% Tween 20), incubated with anti-rabbit IgG-HRP (1:1000 dilution) for 1 h and visualized using DAB as substrate.

Effect of Caspase-3 Inhibitor in Cr(III)-induced Apoptosis

Lymphocytes have been preexposed to 50 mM of N-benzyloxycarbonyl-asp-glu-val-asp-fluoromethylketone (z-DEVD-fmk) a caspase-3 inhibitor for 18 h and then post-exposed to **1**and **4** for 48 h. [^{3}H]-Thymidine incorporation, DNA fragmentation and flow cytometric analysis and Western blotting of the whole cell lysate for investigating caspase-3 activation was carried out employing procedures described earlier.

Results

Chromium Mediated Non-enzymatic Phosphorylation of BSA and Depletion of ATP in Human Lymphocyte Cell Cultures

A marker protein, bovine serum albumin, has been employed to investigate possible role of chromium(III) in non-enzymatic phosphorylation in biosystems. Whereas complexes **1-4** do not seem to elicit any potential to participate in non enzymatic phosphorylation of BSA under the experimental conditions, the treatment of BSA with ATP in the presence of aquapentamminechromium(III) seems to result in the transfer of labelled phosphorous to BSA. SDS-PAGE followed by radio-assays on a reaction product of BSA-ATP and **5** provide direct evidence for the transfer of labelled phosphorus to the protein.

The extent of phosphorylation realized under various experimental conditions are presented in Fig. 1. Quantitative assays reveal that the extent of ^{32}P label transferred to the protein is 0.75 mol per mol of BSA under favourable conditions. On the other hand, number of moles of chromium bound per mol of BSA is found to be as high as 50-55. These data indicate that the ^{32}P incorporation is more specific to some site in BSA and

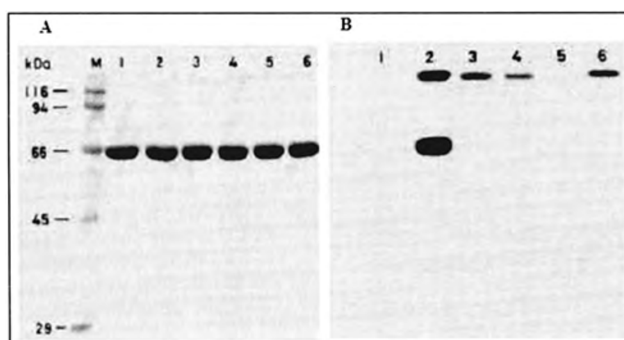
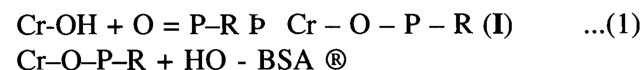


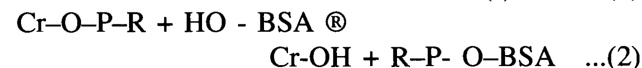
Fig. 1 Phosphorylation of BSA in the presence of different Cr(III) complexes A) CBB stained gel; B) Autoradiogram of the dried gel Lane M, Molecular weight Marker. lane 1, Untreated BSA; lane 2, **5**; lane 3, **1**; lane 4, **2**; lane 5, **3**; lane 6, **4**.

the binding of chromium is not specific to any particular residue in the protein. Comparative assessment of data between ^{32}P labels in alpha and gamma centres with that of ^{3}H labelled adenosine group in ATP reveals that the transfer of ^{32}P label is primarily due to that of phosphate moiety rather than that of adenosine group.

The observation that among the various complexes investigated, only **5** mediates non enzymatic phosphorylation of BSA and the complexes **1-4** do not merit further discussion. Under the conditions of the experiment, complex **5** is known to exist predominantly in its conjugate base form, hydroxopentamminechromium(III), **6**. Previous studies have shown that **6** is capable of undergoing anation reactions with oxo-anions like chromate, molybdate, vanadate, sulfite and phosphate with metal-oxygen bond retention[31]. The observed data on phosphorylation can be rationalized in terms of a reaction mechanism given in Scheme 1.



Cr-OH + O = P–R Þ Cr – O – P – R (**I**) ...(1)



Cr–O–P–R + HO - BSA ®

Cr-OH + R–P- O–BSA ...(2)

(where ATP is represented as O=P-R and HO-BSA represents hydroxyl group in BSA)

Scheme 1

The complexes **1** and **2** do not possess a hydroxo ligand to form intermediates of the type **I** and undergo anation reactions with oxo anions. The complexes **3** and **4** do possess aqua ligands, but previous studies have shown that the metal-oxygen bond breaking in those systems is easy[32,33]. Further, anation processes of complexes **3** and **4** with phosphate groups are expected to render the resulting product anionic and the cationic potential of the metal centre may be inadequate to promote non enzymatic phosphorylation.

That the mechanism involved in chromium(III) mediated phosphorylation may involve the formation of intermediates of **I** is revalidated by means of some competition experiments. Anions like vanadate, molybdate compete with ATP for BSA when phosphorylation is mediated by **6** as evident from data presented in Fig. 2. Whereas complex **6** seems to mediate non-enzymatic phosphorylation, it does not elicit significant potential to cause apoptosis. In other words, the potential to mediate non enzymatic phosphorylation of marker proteins is not related to the ability to cause apoptosis in human lymphocytes.

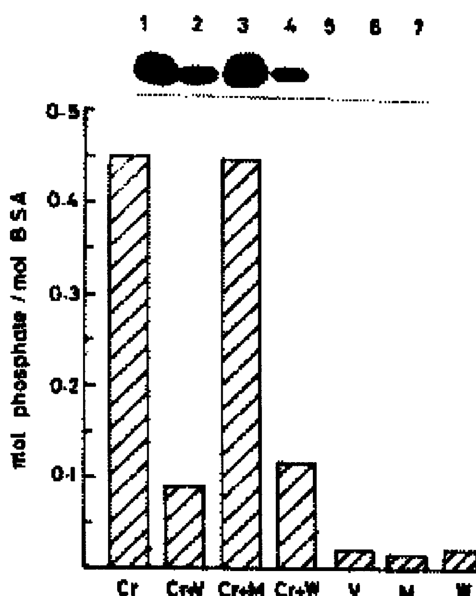


Fig. 2 Competition of oxoanions on the phosphorylation of BSA. Values represent the mean of three separate experiments. (Top) Representative autoradiogram. V, vanadate; M, molybdate; W, tungstate.

The non-enzymatic phosphorylation has been assessed in the present investigation only with a marker protein like BSA. This is not a biologically relevant observation in discussing the apoptosis of human lymphocytes. A direct observation with tyrosine kinases would be necessary for more direct and meaningful conclusions. Experimental constraints precluded such a direct study on phosphorylation of tyrosine kinases. There is no direct evidence for significant depletion of ATP through chromium(III) mediated phosphorylation even in the case of BSA. In the absence of coupling to electron transfer reactions non enzymatic phosphorylation need not lead to significant depletion of ATP. Hence the absence of a significant apoptotic potential for **6** is not surprising.

The complexes **1- 4** cause apoptosis of human lymphocytes but do not seem to participate in non enzymatic phosphorylation of marker proteins like BSA. However, the complexes **1- 4** , cause significant depletion of ATP during the *in vitro* cell cultures of human lymphocytes while **5/6** do not, as seen from the data presented in Fig. 3. It is appropriate to explore also pathways other than non enzymatic phosphorylation in cell biology, to relate Cr(III) induced apoptosis of human lymphocytes with biologically relevant processes.

Assessment of Apoptotic Potentials of Some Select Chromium(III) Complexes on Human Lymphocyte Cells

Proliferation of human lymphocyte cells can be stimulated in *in vitro* cultures using standard and previously known conditions[34]. The effect of some select complexes of Cr(III) (**1-5**) on *in vitro* cell

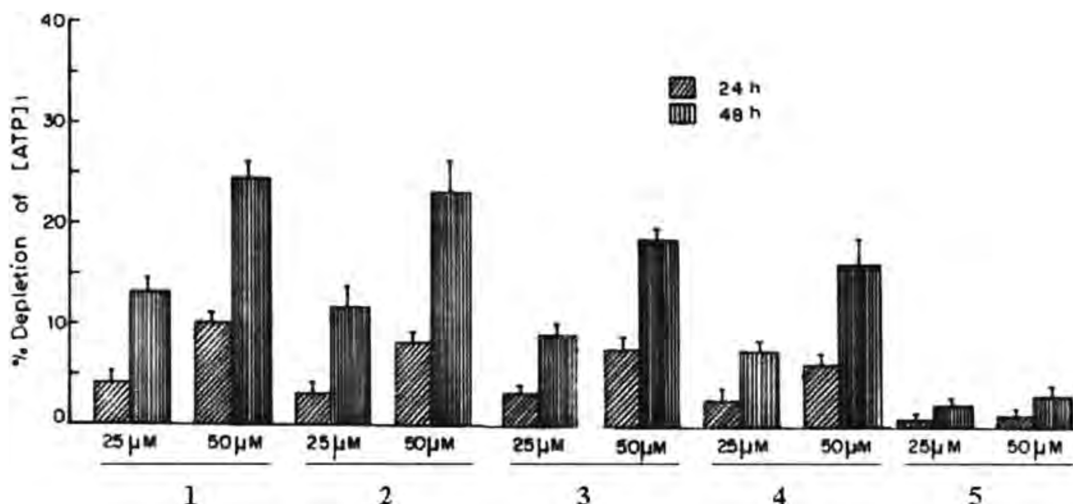


Fig. 3 Intracellular ATP content in lymphocytes during apoptosis in the presence of chromium(III) complexes (**1 – 5**). Values represent the mean ±SE (n = 6)

proliferation of human lymphocytes has been examined. Apoptotic bodies have been observed in some cases, which can be attributed to the effect of the Cr(III) complex added to the culture medium.

Apoptosis is recognized and differentiated from necrosis (un-programmed cell death) in scientific literature by standard methods[35,36]. Employing such standard methods viz. assessment of cell morphology, DNA fragmentation, and flow cytometric analysis, it has been possible to unambiguously assign apoptosis as the mode of cell death caused by the complexes investigated in this study. Based on flow cytometric analysis of cells, it is now possible to analyze at least semi-quantitatively apoptotic potentials induced by the Cr(III) complexes **1-5.** These are presented in Fig. 4. It is evident from the data shown in Fig. 4 that complexes **1-4** induce apoptosis in human lymphocytes and **5** do not. All the complexes **1-4** are known to display potentials to undergo redox conversions to other oxidation states of chromium as known from previous literature on aqueous chemistry of chromium. Reduction of complexes **1** and **2** to Cr(II) forms and oxidation of **3** and **4** to Cr(V) states have been reported earlier[37,38,14]. It is relevant to examine as to whether

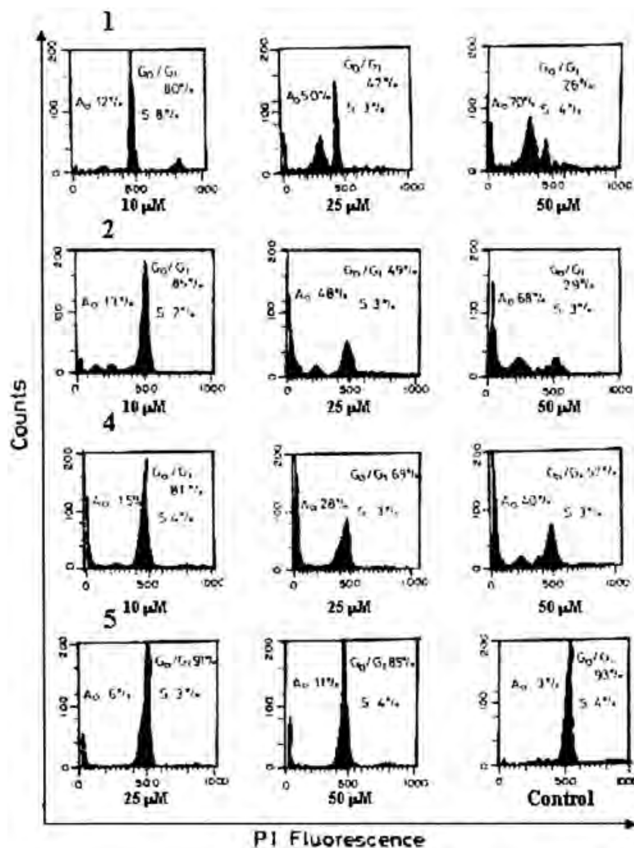


Fig. 4 Measurement of apoptosis by flow cytometric analysis.

such redox conversions in biological systems could in fact promote the formation of ROS.

Potentials of the complexes **1-4** to participate in the formation of ROS under the conditions of experiments in human lymphocyte systems have been investigated directly. Evidence for the possible formation of ROS has been sought from both direct and indirect studies. Extent of ROS formation in the presence of Cr(III) salts has been quantified by means of FACS (Fluorescence Activated Cell Sorter) analysis of both control and Cr(III) treated cells after 3 hrs of incubation. These data are listed in Table I. It is expected that anti-oxidants will react with ROS. If chromium induced apoptosis involved intermediate redox steps and the formation of ROS, a pre-treatment with anti-oxidants will be expected to reduce the apoptotic processes. Such a reduction in apoptosis on pre-treatment with anti-oxidants has been observed in all the cases, where Cr(III) induces apoptosis as seen from the data given in Table II. Reactive oxygen species capable of causing apoptosis have been grouped as superoxide, peroxide and hydroxyl radicals.

Pre-treatment of cells with SOD and catalase seems to reduce the apoptosis potentials of Cr(III) species **1-4.** This is in spite of the potentials for the formation of hydrogen peroxide when inhibited by SOD. Ascorbic acid, a -tocopherol and other free radical scavengers inhibit Cr(III) induced apoptosis. It has been possible to inhibit Cr(III) induced apoptosis using scavengers of free radicals that are generally employed for ROS by other investigators[39]. Changes in hypodiploid DNA levels and the formation DPCs in human lymphocytes have been monitored as a function of chromium(III) induced apoptosis for the complexes **1-5.** Data on apoptotic potentials of **1** - **5** for varying concentrations of Cr(III) are presented in Fig. 5. It can be readily seen that both polypyridyl complexes (**1** and **2**) behave similarly whereas Schiff base complexes (**3** and **4**) behave as one group and **5** does not elicit significant apoptotic potentials.

Chromium(III) Mediated Changes in Expression and Regulation of Src-family of Tyrosine Kinases

Chromium induced changes in tyrosine kinases involved in proliferation of human lymphocytes have been monitored. Proteins synthesized in the presence of Cr(III) compounds have been analyzed for the levels of tyrosine kinases using Western blotting. When stimulated with complexes **1** and **4**, proteins are phosphorylated and molecular weights of the proteins

Table I
Measurement of ROS by FACS Analysis

Treatment	%ROS
Control	23
tris-phenonthroline chromium(III) chloride, **1**	92
tris-bipyridylchromium(III) chloride, **2**	90
Trans diaqua ethylene-bis[salicylaldiminato]chromium(III) perchlorate, **3**	66
trans diaqua propylene bis[salicylaldiminato]chromium(III) perchlorate, **4**	79
Aqua pentamminechromium(III) nitrate, **5**	28

Table II
Effect of Antioxidants on Hypodiploid DNA Content Induced by Chromium(III) Complexes

Treatment	% Apoptosis
tris-phenonthroline chromium(III) chloride, **1**	70
1 + PP2	7
trans diaqua propylene bis[salicylaldiminato]chromium(III) perchlorate, **4**	40
4+ PP2	7

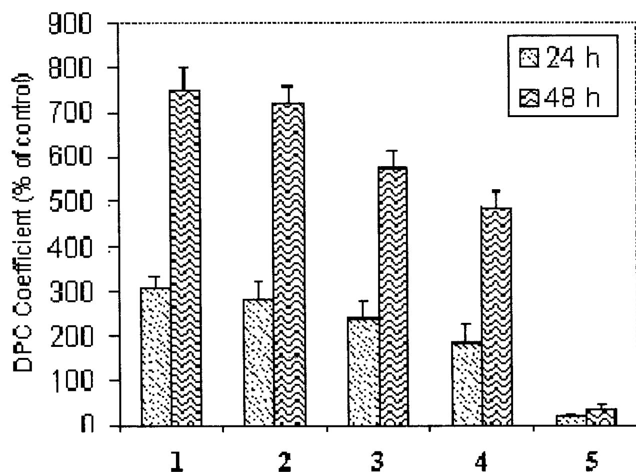


Fig. 5 Formation of DNA-protein cross-links in the presence of Cr(III) complexes. Values reported are the mean ± S.E. of five independent experiments.

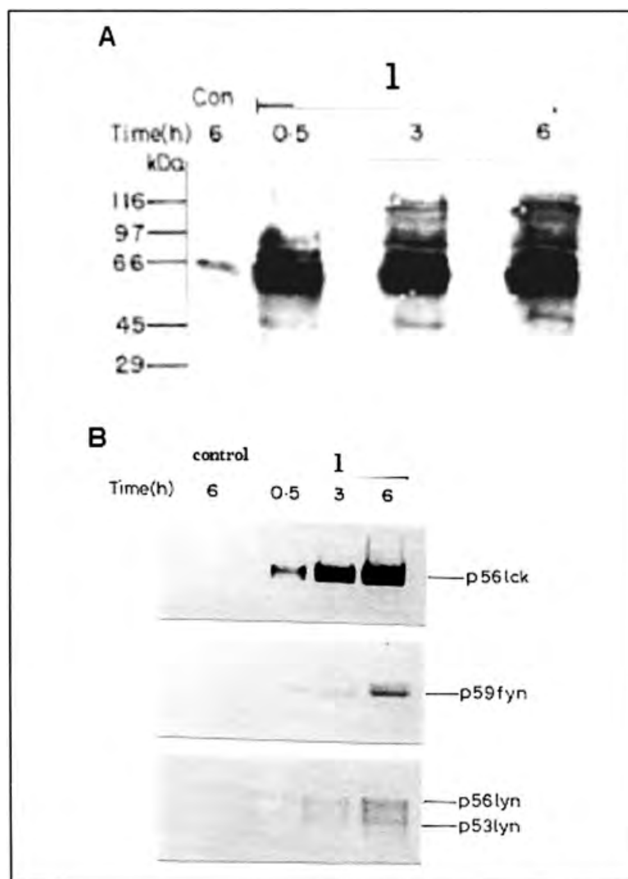


Fig. 6 Signalling pathways activated due to chromium(III) exposure. A. Status of tyrosine phosphorylation in lymphocytes under the influence of **1** B. Expression of Src-family tyrosine kinases under the influence of **1**

that undergo phosphorylation have been estimated as 45,53, 59, 66, 72 and 85 kDa as evident from SDS-PAGE (Fig. 6A).

In order to examine whether some of these phosphorylated proteins in fact belong to the Src family of tyrosine kinases, the cell lysates after immunoprecipitation with anti-phosphotyrosine antibodies, have been probed for polyclonal antibodies specific to p56lck, p59fyn, and p53/56lyn. Since similarities were observed between the effects of **1** with those of **2** and of **3** with those of **4**, Western blotting and other biochemical investigations were limited to the studies on **1** and **4**. In the presence of complexes **1** and **4** expression of some Src-family tyrosine kinases (p56lck, p59fyn and p53/56lyn) is increased on exposure of Cr(III) to varying degrees ranging from 2.5 to 8 times for each group of kinases (Fig. 6B). Activities of Src-family tyrosine kinases in the presence of Cr(III) compounds have been quantitatively estimated. A 12 fold increase of the activity of p56lck, 6 fold increase of p59fyn and 4.5 fold increase of p53/56lyn when compared to controls have been observed. In other

words the expression and up-regulation of activities of Src-family tyrosine kinases (p56lck, p59fyn and p53/56lyn) seem to be activated by those Cr(III) complexes, which express apoptotic potentials in human lymphocytes. It is necessary to show that increased expression and up-regulation of specific tyrosine kinases can be related to apoptotic potentials of Cr(III) complexes. In order to verify this hypothesis, the effect of specific inhibitor 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo(3,4-d)pyrimidine (PP2), for tyrosine kinases on Cr(III) induced apoptosis has been examined. The inhibition of tyrosine kinases by PP2 (at the optimized concentrations of 10mM) inhibits totally the Cr(III) induced apoptosis in human lymphocytes. Synthesis of DNA has been monitored in the presence of **1**. When [Cr(III)] = 25mM, the level of synthesis of DNA is only 50% of that found in control samples. When the cells had been pre-treated with PP2 prior to the exposure to Cr(III) at the same concentration, the inhibition level in DNA synthesis is reduced to 3-4% as against 50% in the case where no PP2 inhibition of tyrosine kinases is employed. Flow cytometric data (Table III) corroborate the reduction of formation of apoptotic bodies when the cells had been pretreated with PP2 prior to the exposure to complexes **1** and **4.**

Table III
Measurement of Sub-G1 Peak in the Presence of PP2

Treatment	% Apoptosis
tris-phenonthroline chromium(III) chloride, 1	70
1 + ascorbic acid	7
1 + GSH	5
1+ a -tocopherol	7
1+ SOD	6
1+ catalase	5
1+ formate	6

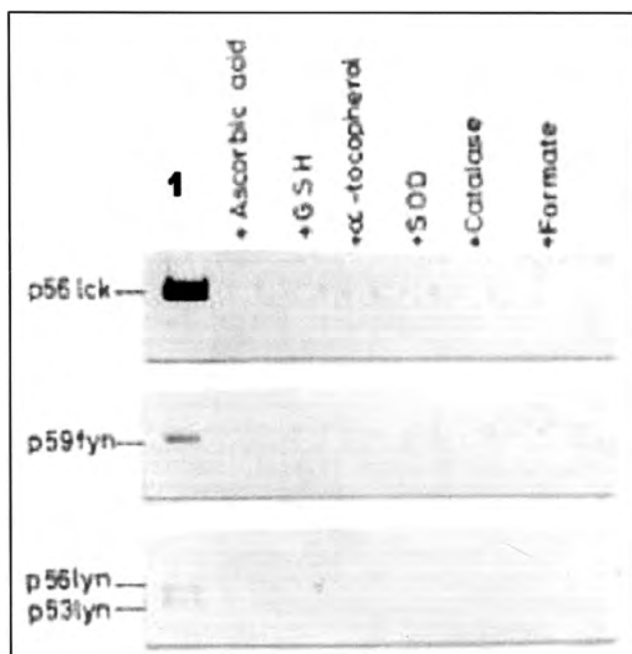


Fig. 7 Activation of Src-family tyrosine kinases depends on *in vivo* ROS.

Western blotting analysis for Src-family tyrosine kinases for cells treated with **1** and **4** have also been carried out after pre-treatment with chemical antioxidants and antioxidant enzymes. When ROS is inhibited by antioxidants even in the presence of Cr(III) complexes, Src-family tyrosine kinases are not activated. This indicates that the ROS generated in the presence of Cr(III) may activate Src-family tyrosine kinases that culminate in the apoptosis of lymphocyte cells (Fig. 7).

Chromium(III) Mediated Activation of Caspase-3 in Lymphocytes

Caspase-3 activation in chromium(III) induced apoptotic systems has been assessed using standard Western blotting technique. It has been observed that 32 kDa procaspase-3 is converted into its active form of caspase-3 (appearance of 17 kDa fragment) after 24 h of treatment of lymphocytes with **1** and **4** and that activity of caspase-3 is sustained for 48h (Fig. 8A). However, pre-treatment of lymphocytes with caspase-3 inhibitor, z-DEVD-fmk, for 18h before exposure to 25 mM of **1** or **4** prevents the activation of caspase-3 (Fig. 8B). Decrease in inhibition of percentage of DNA synthesis and decreases in the sub-G1 peak to significantly low levels (4-5%) have been observed after pre-treatment with caspase-3 inhibitor and even after treatment with chromium(III). The fragmentation of DNA is also reversed in cells, which received pre-treatment with caspase-3 inhibitor prior to the addition of **1** and **4** for 72 h at 50 mM (Fig. 8C). Taken together, these results suggest that activation of caspase-3 is involved in Cr(III)-induced apoptosis, which can be inhibited by the use of caspase-3 inhibitor, z-DEVD-fmk without suppressing either the formation of ROS or up-regulation of Src-family kinases with PP2.

Lymphocytes pretreated with free radical scavengers (ascorbic acid, GSH, SOD, catalase and sodium formate) for 18 h and then challenged with **1** and **4** for 48 h show on immunoblotting with caspase-3 antibody. That the activated form of caspase-3 is absent indicates that antioxidants inhibit also caspase-3

Fig. 8 Inhibition of apoptosis by caspase-3 inhibitor. A. Immunoblot representing caspase-3 activation, B. Effect of z-DEVD-fmk on activation of caspase-3 induced by chromium(III), C. DNA fragmentation in the presence of z-DEVD-fmk.

activation (Fig. 9A). An experiment has been conducted to examine whether activation of caspase-3 is related to the processes controlling the up-regulation of Src-family tyrosine kinases. The expression of Src-family of tyrosine kinases was inhibited by a pre-treatment with PP2 prior to exposure to **1** and **4** and the resulting mixture was immunoblotted for an assay for procaspase-3. The immunoblot reveals the presence of inactive 32 kDa procaspase-3 absence of 17 kDa active caspase-3 (Fig. 9B). These results show that by regulating the activities of Src-family tyrosine kinases in Cr(III) induced apoptosis, it is possible to regulate the activities of caspases.

Discussion

Summarizing the observations of this study, it is feasible to relate Cr(III) induced apoptosis to the formation of ROS. Whereas **5** through its conjugate base **6** participates in non-enzymatic phosphorylation of a marker protein like BSA, the Cr(III) complex with relatively inert redox activity does not cause apoptosis

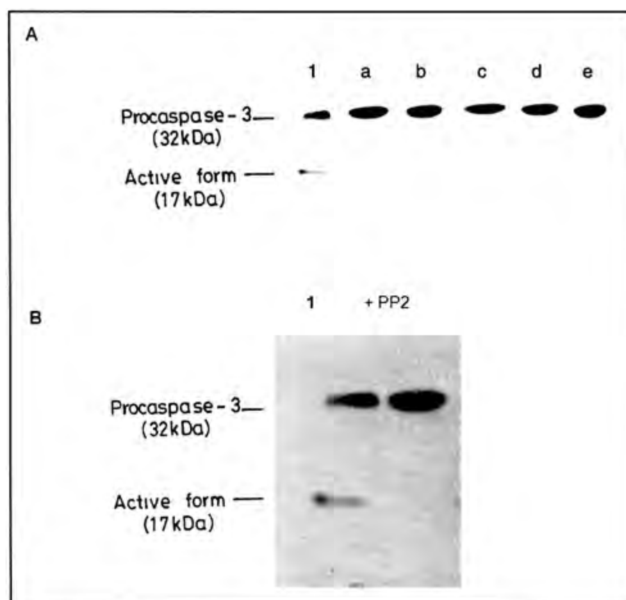


Fig. 9 Immunoblot for caspase-3 activation. A. In the presence of antioxidants, a) + ascorbic acid; b) + GSH; c) + SOD; d) + catalase; e) + formate. B. In the presence of Src-family tyrosine kinase inhibitor

in human lymphocytes. On the other hand, some Cr(III) complexes like **1** - **4**, which could participate in redox pathways promote apoptosis in human lymphocytes. The Cr(III) complexes, typically **1** and **4**, which induce apoptosis, seem to increase also the expression and up regulation of Src-family of tyrosine kinases (p56lck, p59fyn and p53/56lyn) in the absence of free radical scavengers. When the redox state of the cells is changed by the treatment with anti-oxidants or enzymes such as SOD, the increased expression and up-regulation of Src-family tyrosine kinases (p56lck, p59fyn and p53/56lyn) seems to be inhibited almost totally. There is no evidence for parallel pathways for increased expression and up-regulation of Src-family of tyrosine kinases (p56lck, p59fyn and p53/56lyn) through direct reactions with Cr(III) outside the stimulation through ROS mediated processes. Inhibition of Cr(III) stimulated increased expression of Src-family of tyrosine kinases (p56lck, p59fyn and p53/56lyn) by the selective inhibitor PP2, controls also the caspase-3 activation in Cr(III) treated lymphocytes. The results of the present investigation are consistent with a sequence of pathways proposed in Scheme 2.

Cellular Redox partners + Cr(III) ®
ROS + Cr products ...(3)
ROS + Anti-oxidants ® Quenching of ROS ...(3a)
ROS + Tyrosine kinases ®
Up-regulation of Tyrosine kinases ...(4)
Src-family tyrosine kinases + PP2 ®
Enzyme Inhibition ...(4a)
(p56lck, p59fyn and p53/56lyn) + procaspase-3 ®
activation of caspase ...(5)
Activation of caspase ® Apoptosis ...(6)
Procaspase-3 + Z-DEVD-fmk ®
Inhibition of caspase-3 and apotosis ...(6a)

(Where up-regulated tyrosine kinases are p56lck, p59fyn and p53/56lyn)

Scheme 2

A sequential reaction scheme is rationalized in light of the following experimental observations. Chromium induced apoptosis of lymphocyte can be inhibited, when cells are pre-treated with either a free radical scavenger (3a) or PP2 (a selective Src-family tyrosine kinase inhibitor as in 4a) or z-DEVD-fmk (a selective caspase-3 inhibitor as in 6a) prior to exposure to chromium(III) complexes **1** or **4**. The proposed reaction scheme assumes that caspase dependent pathways are down stream processes to Src-family of tyrosine kinase functions. Such an assumption has been supported by previously known pathways in biology of normal cells[40].

No attempt has been made to probe in more detail the redox products of Cr(III) in this study. However, no direct redox reactions of the specific Cr(III) complexes with anti-oxidants are feasible. Apoptotic potentials of complexes **1** – **4** as evidenced from flow cytometric data have been correlated as a function of different concentrations of Cr(III). Plots of $[Cr(III)]^{-1}$ against $[\text{apoptotic potential}]^{-1}$ for the complexes **1-4** are linear with nearly a common intercept (Fig. 10). This may indicate that the apoptotic activity of the Cr(III) complexes may be similar and follow a common mechanism. Slopes of Fig. 10 have been plotted against the percentage of ROS induced by various Cr(III) species(after correcting for the changes in control samples after 3 hrs duration) as evidenced from FACS analyses (Fig. 11). A linear plot in Fig. 11 supports a hypothesis that formation of ROS may be the main pathway through which some of the selected Cr(III) complexes induce apoptosis of human lymphocytes.

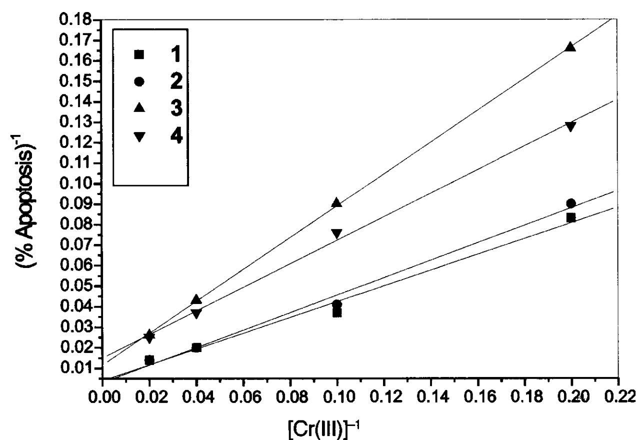


Fig. 10 Plot of $[Cr(III)]^{-1}$ versus $\{\% \text{ apoptosis}\}^{-1}$

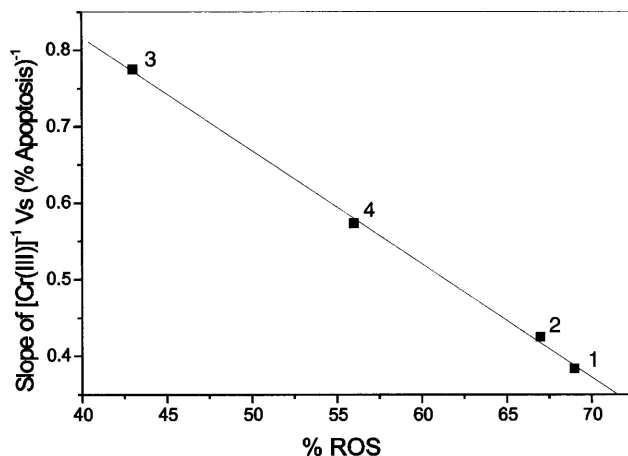


Fig. 11 Plot of slopes $[Cr(III)]^{-1}$ versus $\{\% \text{ apoptosis}\}^{-1}$ for **1** – **4** (obtained from Fig. 10) versus %ROS

Conclusion

Whereas complexes of Cr(III), **1** to **4** cause apoptosis of human lymphocytes, there is sufficient evidence in this study that apoptotic processes induced by the metal ion may be either inhibited or reversed using carefully selected inhibitors. Bio-safety aspects of the clinical use of Cr(III)-picolinate or other derivatives of Cr(III) for control of diabetes need to be critically examined in light of this present study. However, the combined use of such anti-diabetic Cr(III) complexes with anti-oxidants like ascorbic acid may need to be examined.

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