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ROS Induction by Human Calprotectin in K562 and the Reversal Effect of Vitamin E

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Abstract: Calprotectin is a calcium and zinc-binding protein complex that is abundant in the cytosol of neutrophils released under inflammatory conditions. However, the exact role of this factor has not been elucidated. It is composed of 8 and 14 kDa subunits and has the capacity to induce apoptosis in various tumor cells in a zinc-reversible manner. Reactive Oxygen Species (ROS), which are the byproducts of normal cellular oxidative process, regulates the initiation of apoptotic signaling. Recently, it has been shown that calprotectin plays an important role in phagocyte NADPH oxidase activation. In addition, the pretreatment of colon cancer cells with the antioxidant N-Acetyl-L-Cysteine (NAC) prevents apoptosis inducs by calprotectin. In the present study, we further investigate the growth inhibitory effect of calprotectin via ROS induction. For the first time it is shown that human calprotectin induced ROS and apoptosis in K562 cells revealed by conversion of Dichlorodihydroflurescin Diacetate (DCFH2-DA) to DCF and the enhancement of cell surface binding to Annexin V-FITC appropriately. More over, it is demonstrated that naturally occurring antioxidant vitamin E (50-200 μM) significantly reversed the effect of calprotectin proposing the beneficial effect of vitamin E as a natural antioxidant in restriction of calprotectin cytotoxic activity during excessive production of this protein.

Key words: Calprotectin, K562, vitamin E, ROS, apoptosis

INTRODUCTION

Calprotectin, a complex of two calcium-binding proteins belonging to the S100 protein family, is abundant in the cytosolic fraction of neutrophils. A high level of calprotectin reportedly exists in extracellular fluid during various inflammatory conditions, such as rheumatoid arthritis, cystic fibrosis and abscesses. However, the exact biological role(s) of the factor is still under investigation. Recently it has been observed that calprotectin shows growth-inhibitory and apoptosis-inducing activities against various cell types including tumor cells and normal fibroblasts. The findings suggest that calprotectin exerts a regulatory activity in inflammatory processes through its effect on the survival or growth states of cells participating in the inflammatory reaction. It is also possible that calprotectin, at a high concentration, might have a deleterious effect on fibroblasts and influence the recovery of inflammatory tissue. Therefore, the protein factor may be a new drug target to control inflammatory reactions (Yui *et al*., 2003).

In healthy human serum, the concentration of calprotectin is less than 1 μg mL^{-1} while the serum concentrations of calprotectin were 120-fold higher in the initial phase of systemic-onset Juvenile Rheumatoid Arthritis (JRA) compared with healthy controls and approximately 12-fold higher compared with patients with other inflammatory diseases (Frosch *et al*., 2003). An increase in blood calprotectin concentration was also reported in patients with multiple sclerosis (Floris *et al*., 2004) and in fecal of colorectal carcinoma patients was reported (Poullis *et al*., 2004).

Recently, the presence of calprotectin in macrophages and foam cell in human atheroma and in areas of vascular calcification has been reported (McCormick *et al*., 2005). S100A8 and S100A9 mRNA and protein were also expressed by micro-vessels in areas of neo-vascularization. Monomeric and complexed forms of S100A9 were predominant in matrix vesicles isolated from human carotid artery and aorta suggesting a role in regulation of dystrophic calcification. The high amounts of S100A8 and S100A9 complexes in plaque extracts demonstrate the exquisite sensitivity, particularly of S100A8, to oxidation by HOCl. This is strongly suggesting it as a ready target for oxidation *in vivo* (McCormick *et al*., 2005).

As the novel functions of this inflammatory factor are elucidated, the pharmacological manipulation of the factor may open the way to new means of regulating inflammation.

Since calprotectin might cause tissue destruction through its growth inhibitory-or cytotoxic effects under severe inflammatory conditions via induction of ROS, we investigate the induction of ROS and apoptosis by calprotectin in K562 cells then we demonstrate that vitamin E reverses the effect of calprotectin.

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MATERIALS AND METHODS

Materials: Dithiothreitol (DTT) and Lymphoprep were obtained from Merck and Amersham Company, respectively. Fetal Calf Serum (FCS), RPMI 1640 medium, Penicillin, Streptomycin and all other reagents and solvents were purchased from Sigma Chemical Co. and were at least of analytical grade. All solutions were made in double-distilled water.

Preparation of human calprotectin: Fresh heparinized venous blood was obtained from healthy volunteers and leukocytes isolation was performed by dextran sedimentation accordingly (Skoog and Beck, 1956). Granulocytes were re-suspended in Phosphate-Buffered Saline (PBS) containing 0.2 M sucrose, 1 mM EDTA and 1 mM DTT. Cell suspension was sonicated for 30 s, five times by probe type sonicator (Model MK2-3.75, MSE, France). During this procedure the cell container was kept in wet ice. After sonication, the soluble fraction was separated from cell debris by centrifugation at 12000× g for 10 min at 4 °C and clear supernatant (crude leukocyte extract) was collected.

Purification of human calprotectin was done using two steps ion exchange chromatography according to the method previously described (Edgeworht *et al*., 1991). Briefly, the crude leukocyte extract first applied to anion exchange column (Q-Sepharose) and partially purified fractions that showed cytotoxic activity, injected onto cation-exchange column (SP-Sepharose). At this stage calprotectin appeared to be essentially pure with densitometric analysis of Tricine-SDS- PAGE gel that visualized by Coomasie brilliant blue staining. For Tricine-SDS-PAGE previously described method was used (Schagger and Jagow, 1987). Samples were incubated in sample buffer with mercaptoethanol and electrophoresed on acrylamide separating gel (16.5%T, 3%C) with stacking gel (4%T, 3%C) at 200 V.

Protein assay: The protein concentration was determined using the Bradford reagent (Bio-Rad) with Bovine Serum Albumin (BSA) as standard.

Cell line and culture: K562 Chronic myelogenous leukemia, were obtained from cell bank of Pasteur Institute of Iran. These cells were maintained in RPMI 1640 medium supplemented with 10% Fetal Calf Serum (FCS) in a humidified incubator (37 °C and 5% CO2).

The cells were grown in RPMI (pH 7.4), 10% heat inactivated FCS, supplemented with 4 mM L-glutamine, 100 U penicillin and 100 μg mL^{-1} streptomycin in a humidified, 5% CO2 incubator at 37 °C. Harvested cells were seeded into 96-well plates (2×10^4 cell mL^{-1}) and proliferation curves for K562 were determined based on 3-(4,5-dimethtl-2-thiazolyl)-2,5-di-phenyl-2H-tetrazoliun bromide (MTT) assay.

Cytotocicity assay: To evaluate the cytotoxicity effect of calprotectin on K562 MTT colorimetric assay previously described was used (Mosmann, 1983). Briefly, growing cells were transferred into 96-well culture plates containing 200 μL medium alone or medium plus various concentration of calprotectin or medium plus calprotectin and various concentration of antioxidant vitamins and incubated for different time intervals, as indicated, followed by MTT. When antioxidants presented in the culture, four hours before the end of incubations, cells were washed with PBS three times, changed with fresh medium and each well loaded with 25 μL MTT solution (5 mg mL^{-1} in PBS) followed by 4 h incubation. The insoluble formazan produced by living cells were dissolved in a solution containing 10% SDS and 50% Dimethylformamid (DMF) Left for 2 h at 37 °C in dark conditions and OD_{540} was read against blank with multi well scanning spectrophotometer (ELISA reader, Model, Multiskan MS). The percentage of cell viability was calculated using equation: [mean OD_{540} of treated cells mean OD_{540} of control cells] × 100.

Determination of reactive oxygen species production by flow cytometry: K562 cells were cultured in 24 well plates (10^6 cells mL^{-1}, 1mL well) in RPMI-1640 containing 10 % FCS at 37 °C with 5% CO_2 for 24 h. Half an hour to the end of incubation, a final concentration of 10 iM of DCFH2-DA was added and cells were incubated to allow loading of the dye. The cells were then washed three times with PBS and the conversion of non-fluorescent DCFDH2-DA to the highly fluorescent 2-7-Dichlorofluorescein (DCF) was measured using flow cytometer (FACScan, Becton Dickinson, USA).

Activity of cellular esterases cleaves DCFH2-DA into 2-7-Dichlorodihydrofluorescein (DCFH2) and conversion of non fluorescent compound DCFH2 to highly fluorescent compound DCF is occurred in the presence of ROS (Conour *et al*., 2004). Accumulation of DCF in cells may be measured by an increase in fluorescence at 530 nm when the sample is excited at 485 nm. Fluorescence at 530 nm is assumed to be proportional to the concentration of ROS in the cells.

Evaluation of apoptosis (annexin V binding): The cells were plated at a density of 2×10^5 cells $well^{-1}$ with and without calprotectin for 24 h. The tumor cells were harvested and centrifuged at 200 g for 10 min. Cell pellet

was washed with 1X calcium binding buffer and centrifuged at 200 g for10 min. Then 10μL of Annexin V/FITC was added to 1×10^6 cells and incubated for 20 min at 4°C. The cells were washed again with the calcium binding buffer and 10 μL of Propidium Iodide (PI) was added and incubated for 10 min at 4°C. The acquisition and analysis was performed by FACSan (BD, USA) using channels FL1 and FL2 for detection of Annexin/FITC and PI respectively (Sulowska *et al*., 2005).

LC50 Determination: The 50% Lethal Concentration (LC50) of human calprotectin was determined by MTT assay using probit analysis (Pharm program). To determine LC50 of human calprotectin, the 5 concentrations ranging from 0 to 200 μM were used with incubation times at 12, 24 and 36 h. MTT results was analysed for LC50 by probit analysis using the Pharm. PCS statistical package (Springer-Verlage, New York). Statistical differences were determined by Analysis Of Variance (ANOVA) followed with Turkey-Kramer multiple comparison test on the instant package. Differences were regarded as significant at $p<0.05$.

Statistitical analysis: Results were analyzed for statistical significance using two tailed student's t-test. Changes were considered significant at $p<0.05$.

RESULTS AND DISCUSSION

LC50 of human calprotectin against K562: As shown in Fig. 1 treatment of K562 cell line with any concentration of calprotectin for 12 h did not change cell viability but at 24 h 100 μg mL^{-1} can induce death in more than 50% of cells. Since calprotectin concentration in serum and local body fluid was reported to increase in various inflammatory diseases (Roth *et al*., 2001), our observations also allow us to speculate that extra concentration of calprotectin in inflammatory sites may negatively influence the growth or survival state of other cells. In addition it was reported that synovial fluid concentrations of calprotectin in some patients is higher than 100 μg mL^{-1} (Yui *et al*., 2003). This concentration is in the range of calprotectin cell death induction suggesting the possible involvement of calprotectin in joint tissue destruction.

Effect of vitamins E on the cell death inducing activity of human calprotectin: It is still unknown by which mechanism calprotectin initiates cell death signaling. Many cell death inducing pathways are associated with the secondary production of oxidants to contribute the magnitude cell death (Kamal-Eldin and Appelqvist, 1996).

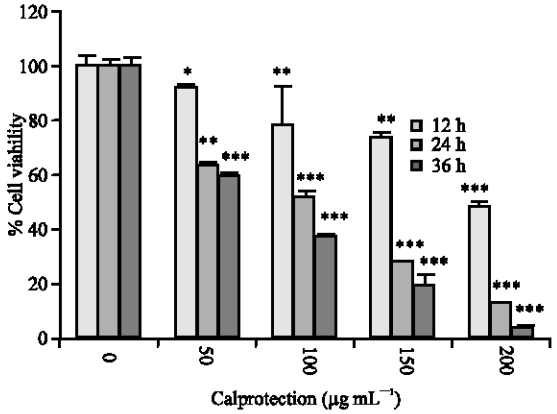

Fig. 1: K562 leukemic cells (2×10^4 cells mL^{-1}) were cultured with varying concentrations of calprotectin sample for 12, 24 and 36 h and cell proliferation was assessed by MTT assay. Results (% Cell viability) are presented relative to cell growth under control conditions (absence of calprotectin) and expressed as percentage of control (as100%).The vertical bars represent S.D. of triplicate determinations and asterisks indicate *$p<0.05$, **$p<0.01$ and ***$p<0.001$ compared to control (absence of calprotectin)

Also some anti-oxidative agents including N-Acetyl-L-Cysteine (NAC) and propyl gallate were reported to inhibit the induction of cell death by several stimuli (Yui *et al*., 2003). We examined effect of natural antioxidant vitamin E to evaluate its potency in neutralization of cytotxic activity of human calprotectin as well as to show the possible involvement of ROS as an essential element in the cell death inducing pathway of this protein. Vitamin E belongs to a family of related compounds known as tocopherols and tocotrienols and from different type of vitamin E; α-tocopherol is typically considered the "gold standard" in the term of antioxidant activity (Kamal-Eldin and Appelqvist, 1996).

As shown in Fig.2, α-tocopherol at all concentrations used in this study, 50-200 μM mL^{-1}, can significantly increase cell viability in a dose dependent manner and concentration of 200 μM markedly inhibits cell death inducing activity of calprotectin in tumor cells. It has been shown that vitamin E increases intracellular Super Oxide Dismutase (SOD) and over expression of SOD reduces cell death (Huang *et al*., 2002). Since it was reported that vitamin C may make vitamin E a more effective antioxidant, by freeing it from α-tocophyryl oxidative free radical (Beyer, 1999; Kadoma *et al*., 2006), we used combination of two vitamins, but no significant synergistic effect was observed (Fig. 3). Vitamin C is an effective water soluble antioxidant commonly present in

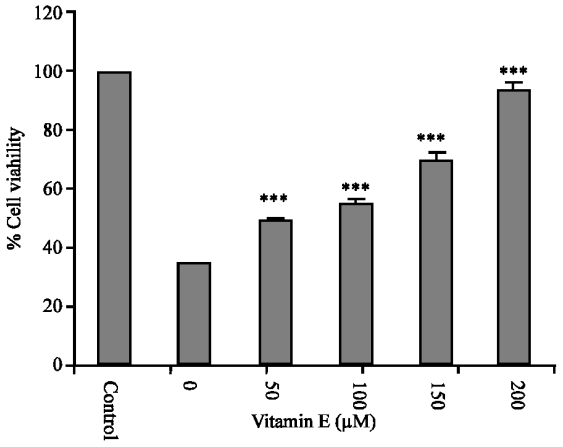

Fig. 2: K562 cells (2×10^4 cells mL^{-1}) were cultured for 24 h without (control) and with 150 µg mL^{-1} of calprotectin, at the presence of various concentrations of vitamin E.. The cell proliferation was quantified based on MTT assay and results are presented relative to cell growth under control conditions (absence of calprotectin) and expressed as percentage of control (as 100%). The vertical bars represent S.D. of triplicate determination and asterisks indicate *p<0.05, **p<0.01 and ***p<0.001 compared to calprotectin alone

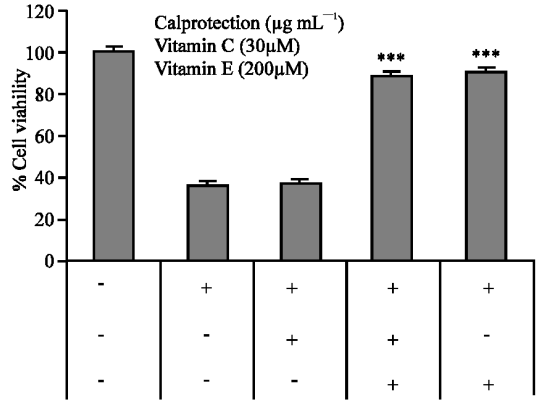

Fig. 3: K562 cells (2×10^4 cells mL^{-})^1 were cultured without (-) and with (+) calprotectin at the presence (+) or absence (-) of two natural antioxidants vitamins E and /or C. After culturing for 24 h, cell proliferation was assessed with MTT assay and results are presented to relative cell growth under control conditions (absence of calprotectin and vitamins) and expressed as percentage of control (as 100%).The vertical bars represent S.D. of triplicate determination and asterisks indicate *p<0.05, ** p<0.01 and ***p<0.001 compared to calprotectin alone

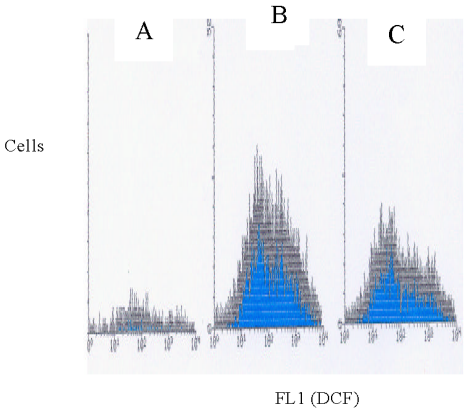

Fig. 4: Cells were cultured without (A) or with 150 µg mL^{-1} human calprotectin (B) and cells were culture with 150 µg mL^{-1} human calprotectin plus 150 µM of vitamin E (C) for 24 h. Then cells were incubated with 10 µM DCFH-DA for 30 min and intracellular ROS contents were measured by flowcytometer

the cytosolic compartment of the cells, directly reacts with ROS and has a vital role against oxidative stress (Carr and Frei, 1999; Meister, 1994). On the other hand vitamin E is the most potent fat-soluble antioxidant in the body that protects cell membranes from damages caused by free radicals (Brigelius and Traber, 1999).

However, vitamin C shows no any synergistic effect on vitamin E at the indicated concentrations and vitamin E appeared to be much more effective than vitamin C in terms of cytoprotectivity.

Detection of ROS in K562 cells: ROS which are the byproducts of normal cellular oxidative processes have been suggested to regulate the process involved in the initiation of apoptotic signaling (Waris and Ahsan, 2006). Fluorescent probes DCFH2-DA is one of the most widely used for direct measuring the redox state of cells. It is a cell permeable, relatively non fluorescent molecule, extremely sensitive to changes in the redox state of a cell. It can be used to follow changes in ROS over time (Conour *et al*., 2004).

In this study, ROS content of the cells was measured directly, using flow cytometry technique and DCFH2-DA as the probe. After 24 h a significant increase in ROS content of treated cells with human calprotectin (150 µg mL^{-1}) was observed. The cells were cultured with both calprotectin (150 µg mL^{-1}) and vitamin E (200 µM) showed partial reduction of ROS content compared to calprotectin treated cells (Fig. 4). The difference of mean fuorescence intensity of control cells with calprotectin treated cells and calprotectin plus vitamin E treated cells were statistically proven using Kolmogorov-Smirnov test

Table 1: Annexin V-FITC/PI cytometry on K562 cell line

	Control	Calprotectin	Calprotectin +vitamin E
% Live cells	88.7±4	27.6±2	98±5
%Apoptotic	3.4±1	20.7±1.9	0
%Necrotic cells	5.4±1.5	15.4±2.1	1.8±0.3
%Late apoptosis cells	2.6±0.9	36.4±3.1	0

These data are representative of three separate experiments

(LYSIS II software, BD, USA). Our results confirmed that calprotectin induces ROS and vitamin E partially inhibits the augmentation of ROS in the cells.

It has been reported that early aggregates have an intrinsic ability to impair fundamental cellular processes by interacting with cellular membranes, causing oxidative stress and increases in free calcium leading to impairment of the cell viability and eventually to cell death (Stefani and Dobson, 2003). Also it has been shown that antioxidants, such as vitamin E protect cells against aggregates toxicity (Stefani and Dobson, 2003). Our previous study also revealed that calcium binding increases surface hydrophobicity of human calprotectin in the range of physiological concentrations and also makes the protein prone to aggregation in the higher concentrations (Yousefani *et al*., 2003). Therefore, we postulated that cell death inducing activity of calprotectin may be governed through toxic pre-aggregation species. This is possible because calcium binding may be lead to formation of a toxic variant of calprotectin with sticky (hydrophobic) surface that interact with plasma membrane to kill cells.

Detection of apoptosis (Annexin V binding): Apoptosis is a physiologically programmed cell death which is essential for the maintenance of normal tissue as well as pathological processes including cancer or inflammation (Bras *et al*., 2005). To quantify the frequency of apoptotic cell induced by human calprotectin, Annexin-V/PI double staining was performed. The cells were treated with the protein (150 μg mL^{-1}) and VP16 (52 μM) for 24 h. Cell surface expression of phosphatidylserine translocation from the inner cytoplasmic membrane is considered an early apoptotic event. Four populations are resolved after Annexin V-FITC/PI staining. Alive cells are double negative detected in the lower left quadrant. Cells that are Annexin V-FITC (+)/PI (-) detected in lower right quadrant are considered as apoptotic. The Annexin V-FITC (-)/PI (+) cell population detected in upper left quadrant are either necrotic or advanced apoptotic. The upper right quadrant, Annexin V-FITC (+)/PI (+), includes cells in late apoptosis. Our data as shown in Table 1 represent that the cytotoxicity of the human calprotectin on the K562 cells is apoptosis based.

Cells treated with human calprotectin for 24 h induced apoptotic population about 20.7±2% compared to untreated cells with 3.4±1% . In presence of vitamin E (150 μM) the apoptotic population of calprotectin treated cells substantially decreased to 0 (Table 1). It is clear that vitamin E has a great inhibitory influence on the apoptosis inducing activity of human calprotectin that probably is correlated with the secondary production of ROS in the target cells.

CONCLUSION

Calprotectin can induce apoptosis in k562 cells and this is clearly due to ROS which accumulates intra-cellular as results of change in redox state. Change in redox state can trigger signal transduction cascade leading to apoptosis. Also these data provide supporting evidences that in addition to zinc and some other transition metals in the body, natural antioxidants such as vitamin E may be important to restrict systemic cytotoxic effect of calprotectin especially where local concentration of calprotectin is relatively high. Neutralization of cytotoxic effects of calprotectin probably prevent tissue destruction.

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