

Cytotoxic effects of human calprotectin on gastric cancer cell line is attenuated by etoposide

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ABSTRACT

Aim: In this paper effect of combinational usage of calprotectin and etoposide on AGS cell line is studied.

Background: Application of combined toxic agents such as etoposide and cisplatin are commonly used for chemotherapy purposes. As a matter of fact, calprotectin and etoposide were both applied on human gastric adenocarcinoma cell line (AGS) as antitumor agents. Both calprotectin and etoposide are topo II inhibitor. Etoposide is a lipophilic agent that can easily transport from membrane while calprotectin active intracellular pathway, probably by membrane surface receptor.

Patients and methods: Calprotectin was purified from human neutrophil by chromatography methods. The human gastric adenocarcinoma cell line was exposed to different concentrations and combinations of calprotectin and etoposide. MTT assay was applied for evaluation of cytotoxicity assay.

Results: Viability of AGS cell line was reduced in high dosages of calprotectin and etoposide. In fact, overnight incubation of these two agents together has been shown less effective than individual usage.

Conclusion: The result indicates that, the combination of both calprotectin and etoposide is considerably less cytotoxic on gastric cancer cells (AGS) than applying individually.

Keywords: Calprotectin, Etoposide, Inhibition proliferation, MTT assay, AGS cell line.

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Introduction

Calprotectin is a calcium- and zinc-binding protein complex composed of 8 and 14 kD subunits. These 8 and 14 kD peptides belong to the S100 protein family which are characterized by two distinct EF-hand calcium-binding motifs with different affinities. Calprotectin inhibits growth of many tumor cells. Calprotectin has cell growth-inhibitory and apoptosis-inducing activity (1-4). It

posses antibacterial and antifungal properties and immunoregulatory activity. It is believed that, calprotectin can link to the cell surface by CD₃₆ receptor (death receptor in stem cells) (5).

In the previous studies have been investigated that calprotectin can inhibit casein kinases which is an important protein in phosphorylation process. Consequently inhibition of casein kinases causes inhibition of important intracellular functions as topo II that is inhibited by annealing breaks of DNA strands. It has been found that some antitumor compounds activate apoptosis in cancer cells (6,7). The chemotherapeutic agents that have been

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identified as apoptosis-inducing include; etoposide, dexamethasone, vincristine, cisplatin, cyclophosphamide, paclitaxel, 5'-fluorodeoxyuridine, 5'-fluorouracil, and adriamycin (7-11). Etoposide is an anticancer agent that is widely used in chemotherapy in patients with breast, lung, prostate and some of gastric cancers. It is a lipophilic agent that can easily transport from membrane and inhibit topoisomerase II and ultimately lead to accumulation of permanent DNA strand breaks in target cells and it happens apoptosis. The efficiency of the antitumor agents seems to be related to the intrinsic propensity of the tumor cells to respond to these agents by apoptosis. This indicates that apoptosis may be the primary mechanism in antineoplastic activity of agents (10-14). Apoptosis is an orchestrated series of events through which the cell precipitates its own death. The stages of apoptosis include cell shrinkage, chromatin condensation, nuclear segmentation and internucleosomal fragmentation of DNA, resulting in the generation of apoptotic bodies (13). Previously we have demonstrated that calprotectin and Etoposide inhibit proliferation and induce apoptosis in AGS cell line (15, 16). In this study we decide to investigate the cytotoxicity assay of combination of calprotectin and Etoposide on AGS cell line.

Methods

Materials

Dithiothreitol (DTT) and lymphoprep were obtained from Merck and Amersham Company respectively. Fetal calf serum (FCS) was obtained from Gibco and Seromed-Germany. RPMI 1640 medium, penicillin, streptomycin, MTT (dimethylthiazol diphenyl tetrazolium bromide) and Etoposide all were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and were at least of analytical grade. In situ cell death detection kit (Annexin-V FITC) was purchased from IQ products-Netherlands. Flask, tubes and culture plates were obtained from Greiner-Germany. Other chemicals used in this study were purchased from Sigma

Chemical Co. All solutions were made in deionized double distilled water.

Cell line

The human gastric adenocarcinoma cell line (AGS, NCBI: C-131) was obtained from National Cell Bank of Iran, Pasteur Institute of Iran. These cells were maintained in RPMI 1640 medium supplemented with 10% FCS in a humidified incubator (37 °C & 5% CO₂).

Calprotectin purification

Human neutrophil were prepared from leukocyte-rich blood fractions (buffy coat) according to the method of Muller et al (17, 18). Method of purification of human calprotectin was described previously (19).

Incubation of calprotectin and Etoposide with AGS for cytotoxicity assay

AGS cells were cultured in RPMI-1640 medium supplemented with 10 % heat inactivated fetal calf serum (FCS), 2mM glutamine, penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37 °C in an incubator containing 5 % CO₂ and 95 % air. Harvested cells were cultured into 96 –well plates (1×10⁴ cell/ml). AGS cells were treated to different concentrations of calprotectin (0, 0.25, 1.025, 2.05, 2.8, 4.1, 6.15 and 8.2 µM) and Etoposide (0, 13, 26, 52, 69.3, 104, 138.6 and 277.2 µM) for 48 hour, then proper concentrations of calprotectins and Etoposide were selected to make a combination of each agents with different concentrations (combination of 52µM Etoposide with 0, 0.25, 1.025, 2.05, 2.8, 4.1, 6.15 and 8.2 µM of calprotectin and combine 2.8µM of calprotectin with 0, 13, 26, 52, 69.3, 104, 138.6 and 277.2 µM of Etoposide) of another agent. Another test was a combination of calprotectin (like above concentration) overnight then treated with the cell line and then finally cytotoxicity was determined. Proliferation curves for AGS cell line was obtained based on the MTT assay.

Viability Test

Relative cell number was measured using MTT assay (20, 21). For MTT assay the dye was dissolved in PBS (Phosphate buffer saline) at a concentration of 5 mg/ml and solution was filtered through a 0.45 μm filter to sterilize and remove small amount of insoluble residues present in some batches of MTT, then stored at 2-8 $^{\circ}\text{C}$ for frequent use. Four hours before the end of incubations, 10 μl of MTT solution (5mg/ml in PBS) was added to each well containing 100 μl cultured medium. Dissolved yellowish MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes at 4 h incubation. In contrast with dead cells, active mitochondrial dehydrogenases of living cells will cause this change. The cleavage and conversion of the soluble yellow dye to the insoluble purple formazan has been used to develop an assay system alternative to other assays for measurement of cell proliferation. The insoluble formazan produced was dissolved in solution containing 100 μl isopropanol and optical density (OD) was read against blank reagent with multi-well scanning spectrophotometer (ELISA reader, Organon Teknika, The Netherlands) at a wavelength of 570 nm. Six AGS cultured wells with 100 μl DDW (deionized distilled water) incubation at 10 min were used as positive control and six AGS cultured wells with no sample were used as negative controls. In addition, six wells containing only dye (100 μl diluted MTT) were used as dye control. The percentage of cytotoxicity was calculated according to following formulas:

$$\% \text{Cytotoxicity} = 1 - \frac{\text{mean absorbance of toxicant-treated cells}}{\text{mean absorbance of negative control}} \times 100$$

$$\% \text{ Viability} = 100 - \% \text{ Cytotoxicity}$$

LC₅₀ Determination

LC₅₀ was determined by probit analysis using the pharm. PCS statistical package (Springer-Verlage, New York).

Statistical analysis

Results were expressed as mean \pm SE. Analysis of data was performed using the Student's *t*-test or χ^2 test. Mean difference between groups was calculated by one and two-way variance analysis. $P < 0.05$ was considered statistically significant.

Results

Antiproliferative interference effect of calprotectin and Etoposide combination on gastric cancer cell line was studied. In the figure 1 the optical density (OD) proportional to the viability of AGS cells that were treated with various concentrations of calprotectin and Etoposide for incubation time of 48 h was determined.

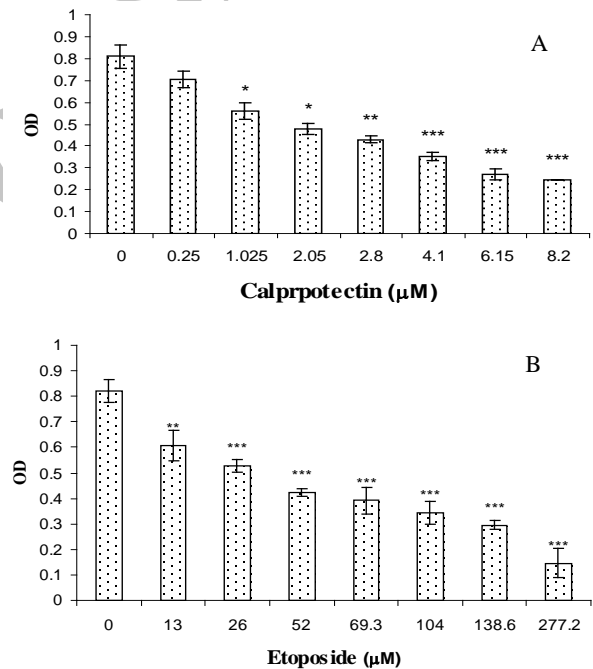


Figure 1. Optical density (OD) expresses as viability rate of different concentrations of calprotectin (A) and Etoposide (B) on cell growth of AGS cells in incubation time of 48h. Results were expressed as the means \pm SD. Statistically significant elucidate by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

The interference effects of combination of calprotectin and Etoposide on the proliferation of

gastric cancer cells were investigated in the figure 2 (A and B). In both figures, calprotectin and Etoposide individually, as control, inhibites cell proliferation in AGS cells in a dosage dependent manner.

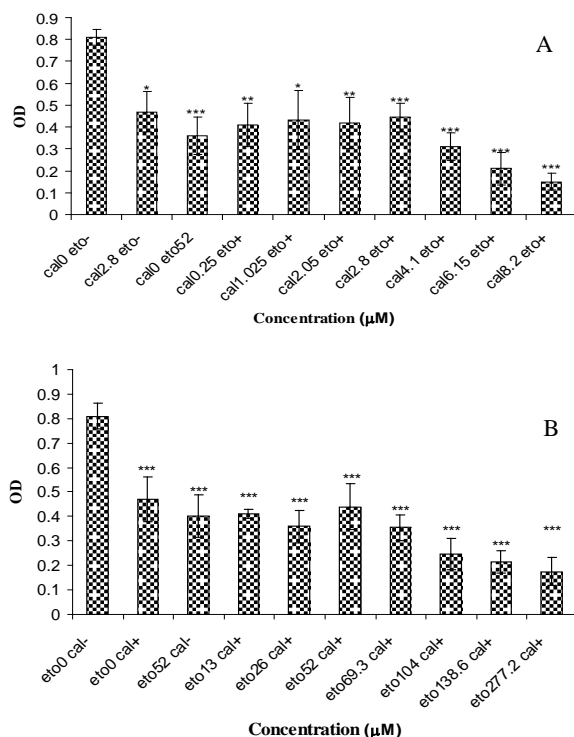


Figure 2. OD of combination of calprotectin and etoposide on the growth of AGS cell line in incubation time of 48 h. A) The cells were treated with combination of different concentrations of calprotectin (0, 0.25, 1.025, 2.05, 2.8, 4.1, 6.15 and 8.2 μM) and fixed concentration (52 μM) of etoposide for 48 h, then the viability was assessed by MTT assay. B) cells were treated with combination of different concentrations of Etoposide (0, 13, 26, 52, 69.3, 104, 138.6 and 277.2 μM) and fixed concentration of calprotectin(2.8 μM). Results were expressed as the means \pm SD. Statistically significant elucidate by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

For appearing more effect LC50 concentrations of Etoposide (52 μM) and calprotectin (2.8 μM) are selected for combinational usage. In their combination used one agent with fixed dosage (in A, Etoposide and in B calprotectin) and other with variable dosages (calprotectin (0, 0.25, 1.025, 2.05, 2.8, 4.1, 6.15 and 8.2 μM) and Etoposid (0, 13, 26, 52, 69.3, 104, 138.6 and 277.2 μM)). In last

experiment before treating the gastric cancer cell with calprotectin and Etoposide, both reagents were combined overnight then treated with AGS cells for 48h which the results are illustrated in the figure 3 (A and B). In figure 3A, over night combination of various concentrations of calprotectin (0, 0.25, 1.025, 2.05, 2.8, 4.1, 6.15 and 8.2 μM) and fixed concentrations of Etoposide (0 and 52 μM) has been indicated.

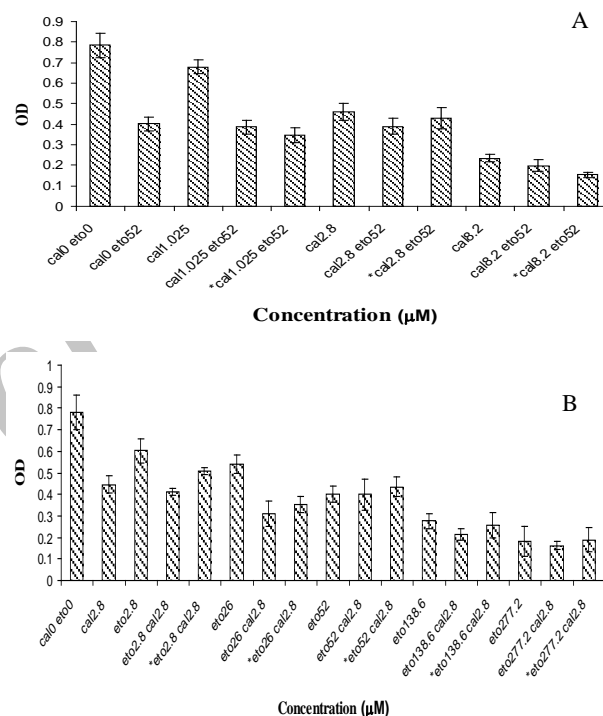


Figure3. OD of overnight combination of calprotectin and etoposide before treatment with AGS cell line for 48 h. A) the cells were treated with combination of different concentrations of calprotectin (0, 0.25, 1.025, 2.05, 2.8, 4.1, 6.15 and 8.2 μM) and fixed concentration (52 μM) of etoposide. B) cells were treated with combination of different concentrations of etoposide (0, 13, 26, 52, 69.3, 104, 138.6 and 277.2 μM) and fixed concentration of calprotectin(2.8 μM). Results were expressed as the means \pm SD. Statistically significant elucidates by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$

Discussion

Calprotectin and Etoposide inhibit the growth of gastric cancer cells in dosage dependent

manner. As depicted in figure 1, treatment of AGS cells with human calprotectin resulted in significantly reduced cell viability at concentrations higher than 1.025 μM while, Etoposide showed significant cell death at all concentrations within 48 h ($P < 0.05$). Here the LC50 parameter that expresses the concentration of agent responsible for decreasing of viability to 50% can be calculated. The LC50 value of calprotectin and Etoposide for AGS cell at 48 h was 2.8 and 52 μM , respectively. Our previous study illustrated that calprotectin inhibits proliferation of AGS cell line about 20 times stronger than Etoposide (2), so in this study try to discover interference effect of these two agents on viability of cells. Figure 2 presents the anti-proliferation effects of calprotectin and Etoposide combinations on the growth of AGS cell line in 48 h. In figure 2-A the cells were treated with combinations of different concentrations of calprotectin and fixed concentration of Etoposide. The findings are not similar to cytotoxicity pattern that illustrated in figure 1-A. It is clear that; the decrement of cytotoxicity is accompanied with increasing of calprotectin concentration by 2.8 μM . It is expected that combination of calprotectin and etoposid show synergism effect of cytotoxicity but possible occurrence of an interaction between them or competition process maybe cause of decreasing cytotoxicity of first phase before 2.8 μM concentration of calprotectin. In low concentrations of calprotectin probably most of Etoposide bind to calprotectin. In the second phase of the experiment, with gradually increasing of calprotectin concentrations, more cytotoxicity occurred that may be due to the presence of calprotectin. This result was repeated in figure 2-B, so can conclude that exist interference pattern of cytotoxicity effect of both agents that exactly related to binding interaction.

For finding interacting behavior of calprotectin and Etoposide established overnight combination

of them before treat with AGS cell line. In fig.3-A and B depicted that the overnight combination of both agents cause the more viability of cells, so it is confirm that Etoposide bind to calprotectin. Previous studies revealed that calprotectin and etoposid have susceptibility to bind with other molecules. Calprotectin is a calcium- and zinc-binding protein complex composed of 8 and 14 kD subunits. Calprotectin displays metal-binding properties that can bind to Ca^{2+} , Zn^{2+} and Cu^{2+} . Therefore, there is considerable variation in binding the metals and biochemical with different physiological consequences (1,4). Etoposide as one of the few anticancer drugs also extensively (>90%) bound to plasma proteins. Subunit of calprotectin characterized by two distinct EF-hand calcium-binding motifs with different affinities. Since These EF-hands are flanked by hydrophobic regions, responsible for interaction with target proteins, and separated by a central hinge region (4,5). Therefore etoposide which including sugar group in its structure (C29H32O13), may bind in EF-hands positions in calprotectin and eliminate the cytotoxicity properties of this component.

Different studies concluded that the unbound fraction of a drug in plasma generally correlates better with pharmacological effects than does the total drug because primarily free drug is available for membrane transport and interaction with receptors or target molecules (22-25). It was reported that there is a wide inter-patient variability in etoposide plasma protein binding (i.e., % unbound, 6 to 37%) among cancer patients receiving etoposide (26). A multivariate analysis of patient characteristics and etoposide protein binding yielded a mathematical model using total bilirubin and albumin to predict % unbound, which explained 93% of the inter-patient variability in etoposide protein binding. Subsequent in vitro studies have confirmed that the binding ratio of etoposide is directly related to

albumin concentration and that etoposide is displaced from albumin by bilirubin, providing a physiochemical basis for the mathematical model (27). Etoposide plasma protein binding has been shown to be clinically relevant as systemic exposure to unbound etoposide has been correlated with hematological toxicity (28). Analysis of figure 2 depicted that exhibit exiting results like maximum interaction between calprotectin and etoposide approximately calculate in 18 molar ratio whereas minimum one is in 3. This shows that etoposide is not able to interact with calprotectin just when concentration of etoposide is 3 fold than the calprotection alone. So the best result would be achieved when used combination of them is in 3 fold higher concentration of etoposid, because there are relationship between free etoposide concentration and bound etoposide concentration. It can be concluded that there are about 15 sites on calprotectin for etoposide binding. The unbound percentage of etoposide or calprotectin can determine the cytotoxicity ratio in AGS cells. Results of the present study suggest that pharmacodynamics properties of etoposide and calprotection that is related to the total and unbound drug concentrations are in need of more investigations.

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