Study of apoptosis inducing activity of calprotectin on fibroblast cell

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ABSTRACT

One of the prominent types of connective tissue cells is fibroblast that synthesizes and maintains the extracellular matrix of many animal tissues. Previous studies illustrated that calprotectin protein has different cytotoxicity effects on fibroblast cells. Calprotectin is abundant in the neutrophil cytosol; it has growth-inhibitory and apoptosis-inducing activities against various cell types such as tumor cells. The present study tries to introduce mechanism of growth inhibitory effect of calprotectin on human foreskin fibroblast cells (HFFF) and compare to etoposide (chemotherapy agent as control). Calprotectin was purified from human neutrophil by chromatography methods. HFFF cell lines were used, maintained in RPMI 1640 medium supplemented with 10% FCS in a humidified incubator (37 °C & 5% CO2). The HFFF cells were exposed to the different concentrations of calprotectin and etoposide for 24, 48 and 72 hours. Cell proliferation was assessed using dimethylthiazol diphenyl tetrazolium bromide assay. Flow cytometric analysis was performed to evaluate the cytotoxic mechanism of calprotectin on HFFF cells. Our results revealed that calprotectin and etoposide induce growth inhibition of HFFF in dose- and time-dependent manners. Sensitivity of HFFF cells to cytotoxic effect of human calprotectin was highly remarkable. In addition, growth inhibitory effect of this cytotoxic agent mostly was governed through induction of apoptosis in the HFFF cells. Taken together, calprotectin not only has more potent anticancer activity in comparison with the etoposide, but it also is an apoptosis inducer that acts on the proliferation of normal cells like fibroblasts.

Keywords: Human Calprotectin; Etoposide; Apoptosis Activity; Flowcytometry; Fibroblast.

INTRODUCTION

Calprotectin is a heterodimeric protein complex with zinc and calcium binding capacity. It is predominantly found in cytosolic component of neutrophils [1-4]. Calprotectin exhibits growth inhibitory and apoptosis inducing activity against some normal and a broad spectrum of tumor cells with different origins; i.e., MM46 mouse mammary carcinoma, MH-134 mouse hepatoma, EL-4 mouse thymoma, L-929 mouse fibrosarcoma, B16 mouse melanoma, J774.1 mouse macrophage-like cells, Ros17/2.8, rat osteosarcoma, MCF-7 human mammary adenocarcinoma, MOLT-4 human leukemia cells and AGS gastric adenocarcinoma cell [1, 2, 5-7]. Several reports suggest that cell death inducing activity of calprotectin is due to exclusion of zinc from target cells [8, 9], and may obey single target single hit theory via binding to its receptor [1]. However, previous studies demonstrate that calprotectin inhibits the activity of casein kinase II, which is involved in the phosphorylation of several enzymes including topoisomerase I and II [10, 11]. Etoposide has been clinically used for more than two decades and is remained one of the most highly prescribed anticancer drugs in the world [12]. It is used as an apoptosis inducer in a variety of cells including breast, lung, prostate and some of gastric cancer cell lines [13-15]. It has been reported that etoposide also has deleterious effects on normal cells [16]. In a previous study cytotoxicity effects of calprotectin on two different human fibroblast cells were compared. It has been investigated that calprotectin has different effect on two
different kinds of fibroblast, but its effect on melanoma cancer cells compared to the fibroblast was further. [17]. A fibroblast is a type of cell that synthesizes and maintains the extracellular matrix of many animal tissues [18]. When a tissue is injured, the fibroblasts nearby proliferate, migrate into the wound and produce large amounts of collagenous matrix which helps to isolate and repair the damaged tissue [19]. As mentioned above about cytotoxicity effect of calprotectin on fibroblast, in this study, apoptotic effects of calprotectin on human fibroblast cell were evaluated and compared with etoposide.

MATERIALS AND METHODS

Dithiothreitol (DTT) and lymphoprep were obtained from Merck and Amersham Companies, respectively. Fetal calf serum (FCS) was obtained from Gibco and Seromed-Germany. RPMI 1640 medium, penicillin, streptomycin, MTT (dimethylthiazol diphenyl tetrazolium bromide) were all purchased from Sigma Chemical Co. 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 Griner-Germany. Other chemicals used in this study were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solutions were made by deionized double distilled water.

Cell line. Human fetal foreskin fibroblast (HFFF-PI6, NCBI: C-170) 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 ºC & 5% CO2).

Calprotectin purification

Human neutrophils were prepared from leukocyte-rich blood fractions (buffy coat) according to the method of previous works [20, 21]. Method of purification of human calprotectin was described previously [21]. Calprotectin was purified from Q sepharose and SP sepharose chromatography. The SDS page electrophoresis gel confirms protein purity.

Incubation of calprotectin and etoposide with HFFF cells

HFFF 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 ºC in an incubator containing 5 % CO2. Harvested cells were seeded into 96-well plates (1x10^4 cell/well) and incubated with the different concentrations of calprotectin and etoposide (0, 1.025, 2.05, 4.1, 8.2 and 16.4µM) for 24, 48 and 72 h. For each concentration of drugs, six wells of 96-well plates containing 1x10^4 HFFF cells were used. In each experiment, six HFFF cultured wells with no drug were used as negative controls. The cultured medium was controlled every day.

Viability Test

Relative cell number was measured using MTT assay (dimethylthiazol diphenyl tetrazolium bromide) [22].

The percentage of cytotoxicity was calculated according to following formulas:

\[ \% \text{Cytotoxicity} = 100 \left(1 - \frac{A_T}{A_N}\right) \]  

That \( A_T \) and \( A_N \) are mean absorbance of toxicant-treated cells and mean absorbance of negative control cells respectively.

\[ \% \text{Viability} = 100 - \% \text{Cytotoxicity} \]  

\( \text{LC}_{50} \) Determination

\( \text{LC}_{50} \) was determined by probit analysis using the pharm. PCS statistical package (Springer-Verlage, New York).

Flow cytometry analysis

For flow cytometry analysis, HFFF cells were cultured into 6-well plates at a density of 5 x 105 cells with and without the cytotoxic agents at 18, 36 and 48 h. All floated and adherent cells were harvested and centrifuged at 200 xg for 10 min. Cell pellet was washed with 1X calcium binding buffer and centrifuged at 200 xg for 10 min. Ten microliters of Annexin V/FITC was added to 100 µl of cell suspension containing 10^6 cells, and incubated at 4°C for 20 minutes. Then, the cells were washed again with the calcium binding buffer and 10 µl of propidium iodide (PI) was added and incubated at 4°C for 10 min and analysis was performed by a flow cytometer (Bio-Rad, USA). FL1 and FL2 channels were used for detection of Annexin/FITC and PI, respectively.
cytotoxicity (LC50) (38 µM) and LC50 of etoposide (63 µM) were selected in evaluation of apoptosis using flow cytometry.

For each experiment of each drugs, 2 well of 6-wells plates of 5 × 10^5 HFFF cells were used and each experiment was repeated 2 times.

Statistical analysis

Descriptive results for quantitative variables were expressed as mean±SD. Analysis of data was performed using the Student's t-test or χ² test. Mean difference between groups was calculated by one and two-way analysis of variance. P<0.05 was considered statistically significant.

RESULTS

The effects of etoposide and human calprotectin on the HFFF cell proliferation

The results of cytotoxicity effect of etoposide and calprotectin on HFFF cells in different concentrations and time intervals (24, 48 and 72 h) are shown in the Figures 1 and 2 respectively. The results of Figures 1 and 2 show that cytotoxic effects of etoposide and calprotectin on HFFF cells are dose and incubation time dependent. Proliferation inhibitory affecting of human calprotectin and etoposide were significant with all concentrations and all time intervals except for 1.025 µM at 24 h incubation time (P<0.001 by one-way ANOVA). Results are expressed as percentage of viability compared to control and are presented as mean±SD. Significance levels by one way ANOVA are *: P<0.05; **: P < 0.01; ***: P< 0.001 that calculated from comparing with control and every concentration.

Measuring apoptotic cell induction

To quantify the frequency of apoptotic cells induced by human calprotectin, Annexin-V/PI double staining was performed. The cells were treated with calprotectin (38 µM and etoposide (63 µM) (positive control) for 18, 36 and 48 h. Cell surface expression of phosphatidylserine (PS) translocated from the inner cytoplasmic membrane is considered an early apoptotic event. By treating cells with either etoposide or calprotectin and then analyzing with flow cytometry, four populations are resolved.

Alive cells are double negative and are seen in the lower left quadrant [A3]. Cells that are Annexin V-FITC (+)/PI (-) [A4] are apoptotic. The Annexin V-FITC (+)/PI (+) cell population [A2] has been described as necrotic or advanced apoptotic. The last quadrant [A1] Annexin V-FITC (-)/PI (+) may be bare nuclei, cells in late necrosis, or cellular debris. Our data represent that the cytotoxicity of both calprotectin and etoposide on the HFFF cells occurs through apoptosis. The apoptotic index values were determined using the flow cytometry results and have been shown in Table 1 and representative Figure 3.
Figure 3. Flow cytometry analysis of the effect of human calprotectin on HFFF cell and compare with etoposide (positive control) and normal cell (negative control) after 18, 36 and 48 h incubation (A, B and C, respectively). Viable cells or Annexin-FITC (-) /PI (-) [A3] are seen in the lower left quadrant. Cells that are Annexin V-FITC (+) / PI (-) [A4] are apoptotic (lower right). The cell population with Annexin V-FITC (+)/PI (+) [A2] has been described as necrotic or advanced apoptotic (upper right) and Annexin V-FITC (-)/PI (+) [A1] may be bare nuclei, cells in late necrosis, or cellular debris (upper left).

Table 1. Apoptotic index (n = 3) of the HFFF cells treated with human calprotectin (38µM) and etoposide (63 µM) for different incubation times. Results were expressed as the means ± SD.

<table>
<thead>
<tr>
<th>Agents</th>
<th>Cells (%)</th>
<th>18 (h)</th>
<th>36 (h)</th>
<th>48 (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Necrotic</td>
<td></td>
<td>13.38±4.50</td>
<td>13.68±2.34</td>
<td>11.17±2.21</td>
</tr>
<tr>
<td>Late apoptotic</td>
<td></td>
<td>22.71±4.13</td>
<td>2.76±0.65</td>
<td>13.34±3.03</td>
</tr>
<tr>
<td>Alive</td>
<td></td>
<td>42.52±3.63</td>
<td>65.39±5.54</td>
<td>40.93±7.04</td>
</tr>
<tr>
<td>Early apoptotic</td>
<td></td>
<td>21.39±2.53</td>
<td>18.17±4.65</td>
<td>3.68±1.03</td>
</tr>
</tbody>
</table>
DISCUSSION

Fibroblasts are derived from primitive mesenchyme and synthesize and maintain the extracellular matrix of many animal tissues (23). Fibroblast as a kind of normal cell with high capacity in proliferation plays a central role in the support and repair of almost every tissue and organ, and the adaptability of their differentiated character is an important feature of the responses to many types of damage. Here, Inhibitory effects of calprotectin and etoposide on HFFF cell lines were compared. Etoposide is an important chemotherapeutic agent that is used frequently to treat a wide spectrum of human cancers [24-28]. As it is shown in the Figure 1, the result of treating cultured HFFF with etoposide shows that this drug has an inhibitory effect on the fibroblast proliferation. Figure 2 represents the inhibitory effect of calprotectin on the fibroblast proliferation. Comparing the effect of calprotectin with etoposide indicates that the same concentration (16.4 µM) of calprotectin and etoposide has a similar effect on the fibroblast viability at the 24 h, but calprotectin decreases the cell survival at the 72 h, two times more than etoposide. So, cytotoxicity effect of calprotectin on fibroblast depends on time. Therefore, in longer incubation time of calprotectin with fibroblast cells has a further cytotoxic effect than etoposide. A previous study demonstrated that calprotectin compare to etoposide has more potent cytotoxicity effect on cancer cells [29]. The 50% LC50 values of human calprotectin and etoposide on HFFF cells at 48 h are 38 and 63 µM respectively. The mechanism of cell apoptosis stimulated by calprotectin has not been fully elucidated. It has been reported that calprotectin may induce apoptosis of multiple tumor cells and consequent inhibition of carcinogenesis by inhibition of casein kinase I and II that inhibit phosphorylation of topo II [30] and activating caspases [31]. Previous studies demonstrated that calprotectin inhibits the activity of casein kinase II, which is involved in the phosphorylation of several enzymes including topo I and II [32]. Because the inhibitors of topo activity induce cell death through apoptosis in various cells [33], it is possible that cell death inducing activity of calprotectin is governed somehow by regulation of topo activity in target cells. The ability to simultaneously measure multiple parameters on a cell by cell basis is probably the most powerful aspect of analytical flow cytometry. This allows flow cytometry to be used for a wide range of applications. Perhaps the most common use is the identification of the presence of antigens either on the surface of or within cells [34]. As it is depicted in Figure 3, cells divided to 4 types of alive, necrotic, early apoptotic and late apoptotic groups. In normal cells, PS residues are found in the inner membrane of the cytoplasmic membrane. During apoptosis, the PS residues are translocated in the membrane and are externalized. In general, though not always, this is an early event in apoptosis and is thought to be a signal to neighboring cells that a cell is ready to be phagocytosed. Annexin-V is a specific PS-binding protein that can be used to detect apoptotic cells [35]. Treatment of the HFFF cells with human calprotectin and etoposide for all times demonstrated that calprotectin caused HFFF cell dead through apoptosis and there are no significant difference in apoptosis induction by etoposide and human calprotectin on HFFF cell line. Other investigators previously determined the apoptotic inducing activity on other kinds of cells (cancer cells) through flowcytometry. Mikami et al. [36] by using flow cytometry showed that calprotectin, induces apoptotic cell death in various tumor cells, suggesting that calprotectin is an effector molecule against tumor cells in polymorphonuclear leukocytes. To explore the cell death-inducing mechanism of the factor, Mikami et al. [36] and Yui et al. [37] confirmed the involvement of target protein synthesis, generation of reactive oxygen species and loss of DNA content in the reaction. Ghavami et al. by using annexin V-fluorescein isothiocyanate apoptosis detection kit showed the treatment of the colon carcinoma cells with different concentrations of human S100A8/A9 induces a significant increase in cell death. Annexin V/phosphatidylinositol and Hoechst 33258 staining revealed that the cell death was mainly of the apoptotic type [31]. The results revealed that calprotectin and etoposide induces growth inhibition of HFFF in a dose- and time-dependent manner. Sensitivity of HFFF cells to cytotoxic effect of human calprotectin was highly remarkable. In addition, growth inhibitory effect of this cytotoxic agent was mostly governed through induction of apoptosis in the HFFF cells.
Taken together, calprotectin has not only more potent anticancer activity in comparison to the etoposide but it also is an apoptosis inducer that acts on the proliferation of normal cell like fibroblast.

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