

Human Calprotectin, a Potent Anticancer with Minimal Side Effect

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Abstract

Background: Chemotherapy by using agents such as etoposide is a common way for inhibition of tumors. This treatment is accompanied by many undesirable side effects. Calprotectin is an abundant protein in the neutrophil cytosol, it has growth-inhibitory and apoptosis-inducing activities against various cell types such as tumor cells. In this study to introduce calprotectin as a suitable substitute anticancer, its growth inhibitory effect on human gastric adenocarcinoma cell line (AGS) and human foreskin fibroblast cells (HFFF) is compared to etoposide effect on these two cell lines.

Methods: Calprotectin was purified from human neutrophil by chromatography methods. AGS and HFFF cell lines were used. These cells were maintained in RPMI 1640 medium supplemented with 10% FCS in a humidified incubator (37 °C & 5% CO₂). AGS cells (10000 cells per well) were exposed to the different concentrations of calprotectin and etoposide for 24 and 48 h. MTT assay was used for evaluation of cytotoxicity.

Results: Results indicate that calprotectin has more potent anticancer activity in comparison to the etoposide but it has nearly similar inhibitory effect on the proliferation of fibroblast cells.

Conclusion: Since calprotectin affect about 20 times more than etoposide on cancer cells, without any additional side effect, it can be concluded that it is a suitable candidate to be studied as anticancer drug.

Keywords: human calprotectin, etoposide, side effect

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Received: 2 Aug. 2009
Accepted: 18 Nov. 2009
IJCP 2009; 4: 183-188

Introduction

Calprotectin is a heterodimeric protein complex with zinc and calcium binding capacity that 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 and MOLT-4 human leukemia cells [1,2,5,6]. Although several reports suggest that cell death inducing activity of calprotectin is due to exclusion of zinc from target cells [7,8], and may obey single target-single hit theory via binding to its receptor [9]. The recent study indicated that the final target of calprotectin may be DNA [10]. By the way, 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 [11, 12]. As the inhibitors of topoisomerases induce cell death through different mechanisms of apoptosis [13, 14], it is possible that the cell death induced by calprotectin is related to regulation of topoisomerase activity in target cells. It was also reported that cytotoxicity of etoposide is governed through abnormal activation of topoisomerase II that ultimately leads to accumulation of permanent DNA strand breaks in target cells [15]. These breaks finally lead to initiation of signaling toward the cell death through apoptosis [16].

Etoposide has been clinically used for more than two decades as one of the most highly prescribed anticancer drugs in the world [17]. It is used as an apoptosis inducer in a variety of cells including breast, lung, prostate and some of gastric cancer cell lines [18-20]. It has been reported that etoposide also has deleterious effects on normal cells [21]. Nausea, vomiting, loss of appetite, diarrhea or

tiredness are common side effects of etoposide and their severity are dose dependent [22]. Therefore, in new drug design try to discover a kind of components with lower side effects.

The wide studies on anticancer properties of calprotectin implies that its cytotoxicity effect on normal cell be studied and compare with etoposide effect, the finding can be interpreted as side effect of two kinds of agents. In this study, the cytotoxic effects of calprotectin and etoposide in various concentrations and time intervals are studied on the AGS and HFFF cell lines to determine the inhibitory intensity of two agents on cancer and normal cell growth.

Materials and 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 at 2005. RPMI 1640 medium, penicillin, streptomycin, trypan blue (TB) and MTT (dimethylthiazol diphenyl tetrazolium bromide) all were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and were of analytical grade. Flask, tubes and culture plates were obtained from Griner- Germany. Other chemicals used in this study were purchased from Sigma Chemical Co. All solutions were made in deionized double distilled water.

Methods

Cell line. Human fetal foreskin fibroblast (HFFF-PI6, NCBI: C-170) and 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 reported method [23, 24]. Method of purification of human calprotectin was described previously [24]. The purification procedure included preparation of human granulocytes, ammonium sulfate precipitation, and anion-exchange chromatography (Q sepharose and SP sepharose) and resulted in the copurification of MRP8, and MRP14. Individual proteins were separated by either preparative isoelectric focusing or preparative SDS-PAGE. The procedure was carried out in the course of 4 days and yielded several milligrams of essentially pure P6, MRP8, and MRP14 in either native or denatured form.

Incubation of calprotectin and etoposide with cell lines. The AGS cells (as a cancer cell line) 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. Harvested cells were seeded into 96-well plates (1×10⁴ 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 and 48hr. For each concentration of drugs, six wells of 96-well plates containing 1×10⁴ cells were used. In each experiment, six cell cultured wells with no drug were used as negative controls. The cultured medium was controlled regularly. For comparing cytotoxicity assay between cancer and normal cells, the HFFF cell line was used.

Viability Test. Relative cell number was measured using MTT assay [25].

The percentage of cytotoxicity was calculated according to following formulas:

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

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

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±SD. Analysis of data was performed using the Student's t-test or x² test. Mean difference between groups was calculated by one and two-way variance analysis. P<0.05 was considered statistically significant.

Results

Etoposide is a chemical agent that is used widely in chemotherapy treatment of cancer [26-30]. In order to analysis of anticancer property of calprotectin, the effect of various concentrations of calprotectin and etoposide (as positive control) at 24 and 48 h incubation times is studied on the AGS cell lines [Fig 1]. The two agents inhibit cell proliferation significantly in all stages of experiment except for 1.025 µM concentration at 24 h time of incubation (P<0.001 by one-way ANOVA). Since a suitable anticancer drug is one that has the least effect on the proliferation rate of normal cells, in the resemble experiment, the effect of calprotectin and etoposide was studied on HFFF (as a normal cell line) [Fig 2]. The obtained results except for 1.025 µM concentration at 24 h incubation time, are statistically significant (P<0.001 by one-way ANOVA).

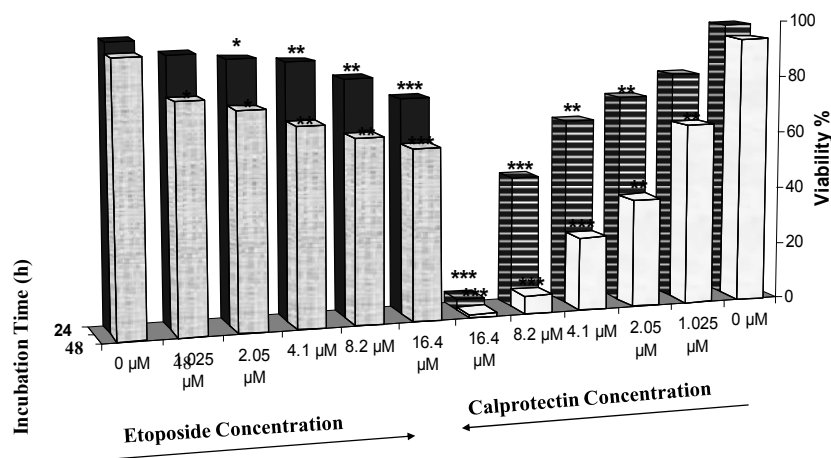


Figure 1. Cytotoxicity measurement of: Etoposide and human calprotectin on AGS cell line proliferation.

The cells were treated with various concentrations of etoposide and calprotectin for 24 and 48 h. The anti-proliferative effect was measured. Results were expressed as mean \pm SD. Significance levels are *: P<0.05; **: P < 0.01; ***: P < 0.001. Inhibition of proliferation was found to be significant at all concentrations of etoposide and calprotectin except for 1.025 μ M concentration of two agents at 24 h incubation time (P < 0.001 by one way ANOVA).

Discussion

Anticancer property of calprotectin on the various cell lines was studied. According to the previous studies that reported anticancer property of calprotectin on the various cell lines, there are three important points regard to the effect of a drug on the human body including potency, mechanism and side effects. The wide ranges of in vitro studies are published in order to description of mechanism of calprotectin effect on the various cancer cell lines [2, 3]. It was also reported that calprotectin inhibits efficiently growth of AGS cell line in a dose and time dependence pathway [31]. In the present study we compared the ability of calprotectin in inhibition the growth of AGS cell line with etoposide (as a common drug in cancer chemotherapy). As it is illustrated in the Fig 1, etoposide has a strong cytotoxic effect on the AGS cell line since viability of the cells decreases continuously during incubation time. Furthermore, we used LC50 parameter to determine the level of cytotoxicity of calprotectin in comparison with etoposide. The LC50 values of calprotectin after 24 and 48 h of incubation times were obtained 3.96

and 1.58 μ M respectively. However the LC50 values of the same incubation time for etoposide were acquired 76.35 and 41.9 μ M respectively. As it is depicted in the Figure 1, it seems that calprotectin has more cytotoxic effect on the AGS cell line in comparison to the etoposide. LC50 ratio of etoposide to calprotectin at 24 and 48 h after incubation were calculated. According to the calculated ratio, it is concluded that calprotectin has strong cytotoxic ability to kill the AGS cells about 19.3 and 26.5 fold at 24 and 48h respectively over etoposide. As it is shown in the Figure 1, 16.4 μ M concentration of calprotectin decreases viability of AGS cell line nearly to zero at 24 h incubation time. Taken together, comparing the LC50 values of calprotectin and etoposide reveals that calprotectin is at least 20 times more potent than etoposide in inhibition of cancer cell proliferation.

The level of calprotectin side effects on the human body was investigated by comparing the cytotoxicity of calprotectin and etoposide on HFFF cell line [Fig2]. Fibroblasts are derived from primitive mesenchyme and synthesize and maintain the

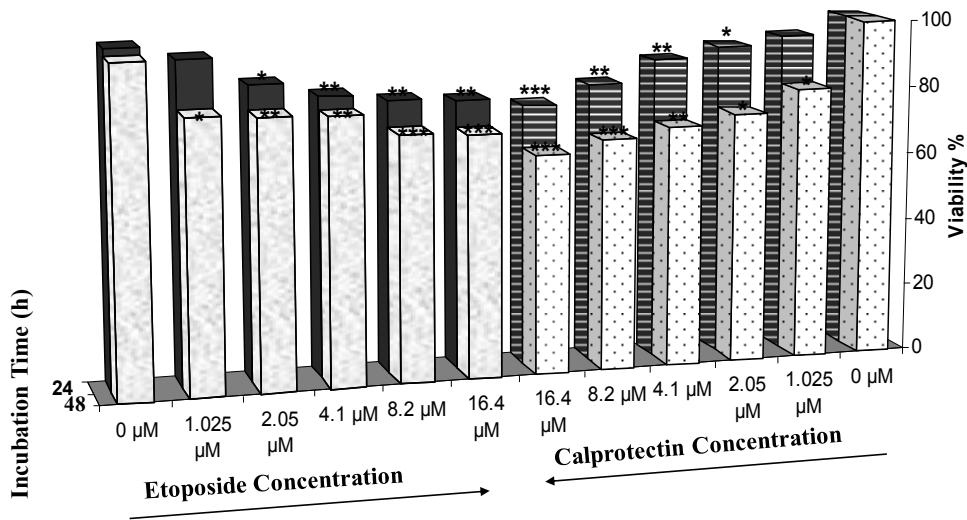


Figure 2. Measuring the inhibitory effect of: etoposide and human calprotectin on HFFF cell line proliferation

The HFFF cells were treated with various concentrations of etoposide and calprotectin for 24 and 48 h. The anti-proliferative effect was measured. Results were expressed as mean ± SD. Significance levels are *: P < 0.05; **: P < 0.01; ***: P < 0.001. Inhibition of proliferation was found to be significant at all concentrations of etoposide and calprotectin except for 1.025 µM concentration of two agents at 24h incubation time (P < 0.001 by one way ANOVA).

extracellular matrix of many animal tissues [32]. Fibroblast is one of the good candidates for studying the side effects of anticancer drugs, because 1) it is a kind of normal cell and 2) it has high capacity in proliferation and plays a central role in the support and repair of almost every damaged tissue and organ. It is important to note that the side effect of calprotectin in comparison with etoposide is not necessarily more; however the inhibitory effect of calprotectin on cell proliferation is about 20 times stronger. According to our experiments [see Fig2], it is reported that both calprotectin and etoposide have the same mild effect on the survival of HFFF cell.

To sum up, calprotectin as a natural product of human body [2] might be adapted cell against cancer and therefore it is reported here as a potent anticancer natural agent with low side effect and it could be introduced as a safe candidate for chemotherapy by additional studies.

Acknowledgment

The financial support from Clinical Proteomics Research Center of Shahid Beheshti Medical University, Skin Research Center of Shahid Beheshti Medical University and Asre-Novin Institute of Research and Industrial Services is gratefully acknowledged.

Conflict of interests

The authors have no conflict of interests in this article.

References

- Shibata F, Ito A, Ohkuma Y, Mitsui K. Mitogenic activity of S100A9 (MRP-14). *Biol Pharm Bull.* 2005; 28:2312-14.
- Yui S, Nakatani Y, Mikami M. Calprotectin (S100A8/S100A9), An inflammatory protein complex from neutrophils with a broad apoptosis-inducing activity. *J Biol Pharm Bull.* 2003; 26: 753-60.

3. Nakatani Y, Yamazaki M, Chazin WJ, Yui S. Regulation of S100A8/A9 (calprotectin) binding to tumor cells by zinc ion and its implication for apoptosis-inducing activity. *Mediators Inflamm.* 2005;24: 280-92.
4. Pakarinen M, Koivusalo A, Natunen J, Ashorn M, Karikoski R, Aitola P, Rintala R, Kolho K. P027 fecal calprotectin mirrors inflammation of the pulled through ileum and bowel function after restorative proctocolectomy for rediatic onset ulcerative colitis. *J Crohn's Colitis Supplements.*2009; 3(1):8.
5. Yui S, Mikami M, Yamazaki M. Induction of apoptotic cell death in mouse lymphoma and human leukemia cell lines by a calcium-binding protein complex, calprotectin, derived from inflammatory peritoneal exudate cells. *J Leukoc Biol.* 1995; 58: 650-658.
6. Champaiboon C, Sappington KJ, Guenther BD, Ross KF, Herzberg MC. Calprotectin S100A9 Calcium-binding Loops I and II Are Essential for Keratinocyte Resistance to Bacterial Invasion. *J Bio Chem.* 2009; 284: 7078-90.
7. Sohnle PG, Hunter MJ, Hahn B, Chazin WJ. Zinc-reversible antimicrobial activity of recombinant calprotectin (migration inhibitory factor-related proteins 8 and 14). *J Infect Dis.* 2000; 182:1272-5.
8. Yui S, Nakatani Y, Hunter M J, Chazin W J, Yamazaki M. Implication of extracellular zinc exclusion by recombinant human calprotectin (MRP8 and MRP14) from target cells in its apoptosis-inducing activity. *Mediators Inflamm.* 2002; 11: 165-72.
9. Zali H, Marashi SA, Rezaei-Tavirani M, Toossi P, Rahmati-Roodsari M, Shokrgozar MA. On the mechanism of apoptosis-inducing activity of human calprotectin: Zinc sequestration, induction of a signaling pathway, or something else? *Med. Hypotheses.* 2007; 68(5):1012-15.
10. Marashi SA, Rezaei-Tavirani M, Zali H, Shokrgozar MA. Mitochondrial DNA might be influenced in calprotectin-induced cell death. *Exper and Clinic Sciences Inter J,* 2008; 7:163-8.
11. Tugizov S , Berline J , Herrera R , Penaranda ME , Nakagawa M , Palefsky J. Inhibition of human papillomavirus type 16 E7 phosphorylation by the S100 MRP-8/14 protein complex. *J Virol.* 2002; 79:1099-1112.
12. Wong EV, Schaefer AW, Landreth G, Lemmon V. Casein kinase II phosphorylates the neural cell adhesion molecule L1. *J. Neurochem.* 1996; 66: 779-86.
13. Chen Y , Su YH, Wang CH, Wu JM, Chen JC, Tseng SH. Induction of apoptosis and cell cycle arrest in glioma cells by GL331 (a topoisomerase II inhibitor). *Anticancer Res.* 2005; 25: 4203-8.
14. Hande KR. Etoposide: four decades of development of a topoisomerase II inhibitor. *Eur. J Cancer.* 1998; 34: 1514-21.
15. Barker CR , McNamara AV, Rackstraw SA, Nelson ED, White MR, Watson AJ, Jenkins JR . Inhibition of Hsp90 acts synergistically with topoisomerase II poisons to increase the apoptotic killing of cells due to an increase in topoisomerase II mediated DNA damage. *Nucleic Acids Res.* 2006; 34:1148-57.
16. McClendon AK, Osheroff N. The geometry of DNA supercoils modulates topoisomerase-mediated DNA cleavage and enzyme response to anticancer drugs. *Biochem.* 2006; 745: 3040-50.
17. Duca M , Guianvarc'h D, Oussedik K, Halby L, Garbesi A, Dauzonne D , Monneret C , Giovannangeli N, Arimondo PB. Molecular basis of the targeting of topoisomerase II-mediated DNA cleavage by etoposide derivatives conjugated to triplex-forming oligonucleotides. *Nucleic Acids Res.* 2006; 34: 1900-11.
18. Friedrich K, Wieder T, Von CH, Radetzki S, Janicke S , Schulze-Osthoff K, Dorken B, Daniel PT. Overexpression of caspase-3 restores sensitivity for drug-induced apoptosis in breast cancer cell lines with acquired drug resistance. *Oncogene.* 2001; 20: 2749-60.
19. Liu S, Wu Q, Ye XF, Cai JH, Huang ZW, Su WJ. Induction of apoptosis by TPA and VP-16 is through the translocation of TR3. *World J Gastroenterol.* 2002; 8: 446-50.
20. Feliu J , Gonzalez MB , Zamora P , Garcia PA , Garcia CG , Blanco E , Belon J , Garrido P , Jara C , Ruiz A , Vincent JM, Espinosa E. Experience of oncopaz cooperative group with oral fluoropyrimidines in tumors of the stomach, lung, head and neck, and breast. *Oncology.* 1997; 54: 30-37.
21. Sortibrán AN, Tellez MG, Rodríguez-Arnaiz R. Genotoxic profile of inhibitors of topoisomerases I (camptothecin) and II (etoposide) in a mitotic recombination and sex-chromosome loss somatic eye assay of *Drosophila melanogaster*. *Mutat Res.* 2006; 604:83-90.
22. Marigny K, Aubin F, Burgot G, Gall E, Gandemer V. Particular cutaneous side effects with etoposide-containing courses: is VP16 or etoposide phosphate responsible? *Cancer Chemother Pharmacol.* 2005; 55:244-50.
23. Muller G, Kerkhoff C, Hankowitz J, Pataki M, Kovacs E, Lackner KJ, Schmitz G. Effects of purinergic agents on human mononuclear phagocytes are differentiation dependent. Implications for athero-genesis, *Arterioscler. Thromb.* 1993; 13: 1317-26.
24. van den Bos C, Rammes A, Vogl T, Boynton R, Zaia J, Sorg C, Roth J. Copurification of P6, MRP8, and MRP14 from human granulocytes and separation of individual proteins. *Protein Expr Purif.* 1998; 13: 313-18.
25. Francis D, Rapid RL. Colorimetric assay for cell growth and survival: modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J Immunol Meth.* 1986; 89: 271-7.
26. Stahl S, Kasimir-Bauer M, Harstrick A. Down-regulation of topoisomerase II by camptothecin does not prevent additive activity of the topoisomerase II inhibitor etoposide in vitro. *Anticancer Drugs.* 1997; 8: 671-6.
27. Aisner J , Musanti R , Beers S, Smith S, Locsin S, Rubin EH. Sequencing topotecan and etoposide plus cisplatin to overcome topoisomerase I and II resistance: a pharmacodynamically based Phase I trial. *Clin Cancer Res.* 2003; 9: 2504-9.
28. Walker PR, Youdal T, Leblanc J, Whitfield JF, Sikorska M. Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Res.* 1991; 51: 1078-85.
29. Seiter K. Toxicity of the topoisomerase II inhibitors. *Expert Opin Drug Saf.* 2005; 4: 219-34.

30. Gullatte MM, Graves T. *Advances in antineoplastic therapy. Oncol Nurs Forum.* 1990; 17: 867-76.

31. Zali H, Rezaei-Tavirani M, Kariminia A, Yousefi R, Shokrgozar MA. *Evaluation of Growth Inhibitory and Apoptosis Inducing Activity of Human Calprotectin on the*

Human Gastric Cell Line (AGS). Iranian Biomed J. 2008; 12 (1): 7-14.

32. Doljanski F. *The sculpturing role of fibroblast-like cells in morphogenesis. Perspect Biol Med.* 2004; 47(3):339-56.