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PJBS

ISSN 1028-8880

**Pakistan
Journal of Biological Sciences**

ANSI*net*

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A Comparative Study of Inhibitory Effect of Human Calprotectin on the Growth of Human Gingival Fibroblast and Foreskin Fibroblast

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Abstract: In the present study, cytotoxicity effects of calprotectin on Human Gingival Fibroblast (HGF) and Human Foreskin Fibroblast (HFFF) were compared. For these evaluations, both cells were exposed to the different concentrations of calprotectin, for 24, 48 and 72 h. Cell proliferation was assessed using MTT assay. Our results revealed that growth inhibition of calprotectin on HGF and HFFF occur in a dose- and time-dependent manner. Results of this investigation showed that sensitivity of HGF cells to cytotoxic effect of human calprotectin was more than HFFF. The results indicate that drug resistance process is different for the two kinds of fibroblast cells.

Key words: Human calprotectin, gingival and foreskin fibroblast cells, growth inhibition

INTRODUCTION

Calprotectin is a calcium binding protein that found mainly in granulocytes. This protein is predominantly found in cytosolic fraction of neutrophils (Shibata *et al.*, 2005; Yui *et al.*, 2003; Nakatani *et al.*, 2005; Poullis *et al.*, 2003). Calprotectin has zinc binding properties and reversible antimicrobial and antifungal activity (Sohnle *et al.*, 2000; Yui *et al.*, 2002) and exhibits growth inhibitory and apoptosis inducing activity against some normal and a broad spectrum of tumor cells with different origins (Shibata *et al.*, 2005; Yui *et al.*, 2003, 1995; Mikami *et al.*, 1999). Since the inhibitors of topoisomerases induce cell death through apoptosis in various cells death mechanisms (Chen *et al.*, 2005; Hande, 1998), it is possible that the cell death induced by calprotectin is related to regulation of topoisomerase activity in target cells. It was expressed that calprotectin induces apoptosis via binding to its receptor and its inhibitory effect on the cell growth obeys single target single hit theory (Zali *et al.*, 2007).

Connective-tissue cells play 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 (Martin, 1997).

A fibroblast is a type of cell that synthesizes and maintains the extracellular matrix of many animal tissues (Doljanski, 2004). Fibroblasts seem to be the least specialized cells in the connective-tissue family. They are dispersed in connective tissue throughout the body and provide a structural framework (stroma) for many tissues and play a critical role in wound healing (Martin, 1997; Doljanski, 2004). They are the most common cells of connective tissue in animals. The main function of fibroblasts is to maintain the structural integrity of connective tissue by continuously secreting precursors of the extracellular matrix, primarily the ground substance and a variety of fibers. The composition of the extracellular matrix determines the physical properties of connective tissues (Doljanski, 2004). 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 (Ducy *et al.*, 2000). Fibroblasts change their character in response to chemical signals (Martin, 1997). One of the main side effects of chemotropic is induction of damages to the epithelial cells (Jiang *et al.*, 2007). Fibroblast has ability to thrive in the face of injury, together with their solitary lifestyle and the main support for regeneration of epithelial layer and responsible for the architectural framework of the body (Martin, 1997). Since patient after

treatment via chemotropic procedure, suffer from injuries, obviously, we think that HGF is more sensitive than HFFF during chemotherapy; therefore, its drug resistance is lower than HFFF. Here; survival of HGF and HFFF cells in the presence of some anticancer agent is studied.

MATERIALS AND METHODS

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 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. All solutions were made in deionized double distilled water.

Cell line: Human fetal foreskin fibroblast (HFFF-PI6, NCBI: C-170), human gingival fibroblast cell line (HGF.1-PI, NCBI: C-165) and human malignant melanoma cell (A375, NCBI: C-136) were 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 and 5% CO₂).

Calprotectin purification: Human neutrophil were prepared from leukocyte-rich blood fractions (buffy coat) according to the method of Muller *et al.* (1993). Method of purification of human calprotectin was described previously (Van den Bos *et al.*, 1998). Calprotectin was purified from Q sepharose and SP sepharose chromatography. The SDS page electrophoresis gel confirms protein purity.

Incubation of calprotectin with cells: Foreskin fibroblast cells were cultured in RPMI-1640 medium supplemented with 10% heat inactivated Fetal Calf Serum (FCS), 2 mM glutamine, penicillin (100 IU mL⁻¹) and streptomycin (100 µg mL⁻¹) 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 (0, 1.025, 2.05, 4.1, 6.15 and 8.2 µM) for 24, 48 and 72 h. For each concentration of drugs, six wells of 96-well plates containing 1×10⁴ HFFF and HGF cells were used. In each experiment, six HFFF and HGF cultured wells with no drug

were used as negative controls. The cultured medium was controlled for detection of no microbial activity every day. HGF and HFFF are normal cells but in spite of the other normal cells have high proliferation rate, therefore their growth properties is resemble to the cancer cell lines in media culture. Melanoma cancer cell line was used for comparing cytotoxicity assay between cancer cells and normal cell.

Viability test: Relative cell number was measured using MTT assay (Francis and Rapid, 1986).

The percentage of cytotoxicity was calculated according to following formulas:

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

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

Statistical analysis: Results were expressed as mean±SD. 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.

RESULT AND DISCUSSION

Previous study (Zali *et al.*, 2008) has revealed that 2.8 µM concentration of calprotectin is the LC₅₀ value for gastric cancer cell (LC₅₀ is the concentration of agent that decreases cell survival to 50% relative to the negative control cells). The growth inhibitory effect of calprotectin and chemical anticancer agents such as etoposide, cisplatin and doxorubicin in the equal concentration (2.8 µM) at 48 h incubation time on the HFFF, HGF and A375 were shown in the Fig. 1. The results of cytotoxicity effect of calprotectin on HFFF in different concentrations and time intervals (24, 48 and 72 h) by using MTT assay were shown in Fig. 2. Figure 3 as like Fig. 2, represents the effect of calprotectin on the HGF cells. Proliferation inhibition by human calprotectin was significant at all time intervals except for the 1.25 µM of calprotectin at 24 h time of incubation. Figure 4 shows the curvature form for the data correspond to the Fig. 2 and 3 at 24, 48 and 72 h times of incubation for HFFF and HGF cells.

As it is shown in the Fig. 1, incubation of HFFF cells and HGF with 2.8 µM concentration of calprotectin (Shokrgozar *et al.*, 2007), etoposide, cisplatin and doxorubicin at 48 h induces cell growth inhibitory effect in the culture cells. However the all treatment have approximately similar inhibitory effect on the two kinds of fibroblast cells, it seems that the sensitivity of the two tissues is equal. Calprotectin is a non chemical anti cancer

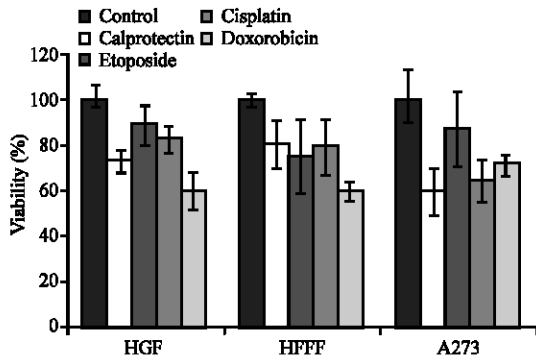


Fig. 1: Measuring the inhibitory effect of human calprotectin, etoposide, cisplatin and doxorubicin; on HFFF, HGF and A273 cell proliferation. Results are expressed as percentage of viability compared to control and are presented as mean±SD

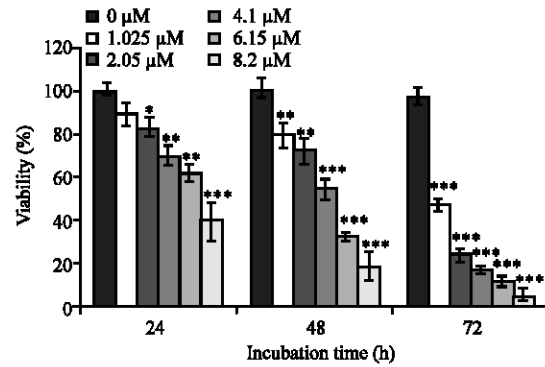


Fig. 3: Cytotoxicity measurement of: human calprotectin on HGF cell proliferation. Results were expressed as mean±SD. Significance levels are *: p<0.05; **: p<0.01; ***: p<0.001. Inhibition of proliferation was found to be significant at all concentrations of human calprotectin (by one way ANOVA)

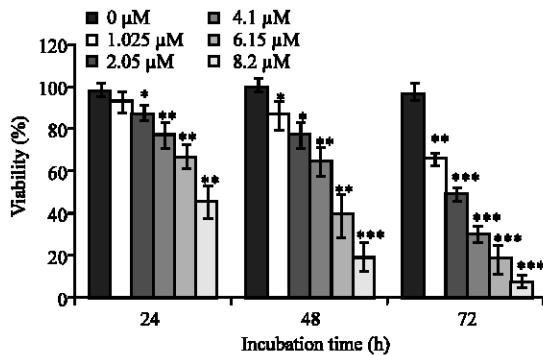


Fig. 2: Measuring the inhibitory effect of human calprotectin; on HFFF cell proliferation, Results are expressed as percentage of viability compared to control and are presented as mean±SD. Significance levels are *: p<0.05; **: p<0.01; ***: p<0.001. Inhibition of proliferation was found to be significant at all concentrations of human calprotectin (by one way ANOVA)

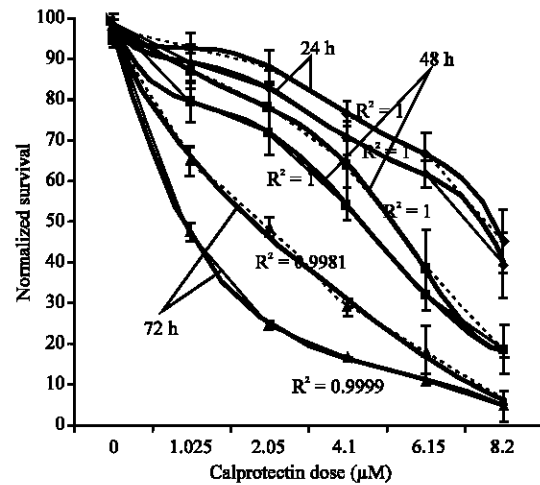


Fig. 4: The curvature form of the data correspond to the Fig. 2 and 3. In three incubation times, the upper and lower curves refer to HFFF and HGF, respectively. The thick lines show the fitted data as trend lines. Regression coefficients (R) for the fitting data are shown beside the related curve in the figure

agent (a human product in the response of body to some disease (Poullis *et al.*, 2003)) acts as like the chemical reagent on the treated cells in the LC₅₀ concentration. The finding of Fig. 1 shows that calprotectin has more inhibitory effect on A273 cell growth relative to fibroblast cells. For better resolution, calprotectin was applied as a cell inhibitory agent and supply to the HFFF and HGF cells. The two cell types were incubated with human calprotectin in the various concentration and time intervals. The results of Fig. 2 and 3 show that cytotoxicity effects of calprotectin on the HFFF and HGF cells are dose and incubation time dependent. According to the results of Fig. 2 and 3, it is possible to assume that

human calprotectin can initiate signaling toward the HGF cell-death with the lower doses in compare to HFFF, especially at 72 h time of incubation. For better understanding, the data of Fig. 2 and 3 were illustrated as curve graphs in Fig. 4. The fitted curves in the Fig. 4 indicate that inhibitory effect of calprotectin for HGF is more than HFFF at all times of incubation especially at 72 h. The comparison of the two curves at 24 h indicated the pattern of survival change for the two cells line is

similar. However, this aspect of study is observed at 48 h time of incubation. As it is depicted in Fig. 4, the patterns of cell survival curves are different for the both cells at 72 h. The curve refer to the HFFF tend to be linear but another curve show significantly curvature, so; different patterns can expresses the more sensitivity of HGF relative to HFFF in the presence of calprotectin at 72 h. However illustration of error bars indicate that it is no difference between sensitivity of two type of fibroblast at 24, 48 h but at all experiments (24, 48 and 72 h), the survival curves of HFFF is upper than HGF. Previous investigations have shown that tumor cells may be drug-resistant, due to the over-expression of some proteins (e.g., P-glycoprotein or multi-drug-resistance associated protein (MRP)) (Keller *et al.*, 2006). It is reported that the normal cells response to the toxin by efflux pump system. These proteins confer resistance to the tumor by pumping the drug out of the cells (Keller *et al.*, 2006; Tomonaga *et al.*, 1996). 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 (Tugizov *et al.*, 2007; Wong *et al.*, 1996). Inhibition of topoisomerases lead to apoptosis (Chen *et al.*, 2005; Tugizov *et al.*, 2007), it is possible that calprotectin induce in similar mechanism. It seems that the responses of two kinds of cells are different. So this effect may be not identical for two kinds of fibroblast cells. By the way, it can conclude that calprotectin affect on both cells by similar mechanism that it may be attenuated by presence of drug resistance system for calprotectin in membrane of HFFF relative to HGF. This study emphasize on the different drug resistance system in two kind of fibroblast that can be study in the future project.

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