Possible Involvement of a Specific Cell Surface Receptor for Calprotectin-Induced Apoptosis in Colon Adenocarcinoma and Carcinam Cell Lines (SW742 and HT29/219)

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Abstract

Calprotectin, a calcium-bound protein complex, is abundant in the cytosol of neutrophils. It has been reported that this protein has an apoptotic activity in tumor cells. Since calprotectin increases in colorectal cancer, this study was conducted to investigate, for the first time, the cytotoxicity/apoptotic effect of calprotectin on HT29/219 and SW742 colon carcinoma and adenocarcinoma cell lines. MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay, annexin V/PI and Hoechst 33258 staining were used to detect apoptotic cells. The activation of caspase-3 and -9 was assayed using caspase assay kits. Flow cytometer was used to determine if there is any binding of calprotectin to its receptor. Treatment of the cells with different concentrations of human calprotectin resulted in a significant increase in the cell death. Annexin V/PI and Hoechst 33258 staining revealed that the cell death was mainly of apoptotic type. A significant ($p < 0.05$) increase in the activity of caspase-3, and -9 was observed in both cell lines following the treatment. However no binding activity was observed. In Conclusion, this study showed that human calprotectin has an apoptotic effect on HT29/219 and SW742 cells, and that the mode of action is caspase activation particularly *via* mitochondrial route.

Keywords: Calprotectin receptor; Apoptosis; Caspase-3 and -9, Colon cell lines

Introduction

Calprotectin is a calcium-bound protein complex composed of 8 and 14 kDa proteins [1]. These proteins, belong to the S100 protein family [2], have also been termed migration inhibitory factor-related proteins (MRP8 and MRP14) [3], calgranulin A and B, or S100A8 and S100A9 [4]. Neutrophils are believed to be a predominant source of calprotectin [5]. The concentration of calprotectin in extracellular fluids

increases under various inflammatory conditions [6-10], suggesting that this factor has important functions in the inflammatory reactions. It has been reported that normal human serum concentration is less than 1μg/ml, but the titers increase in many pathological conditions including cystic fibrosis, rheumatoid arthritis, and Crohn's disease [10]. Very high concentrations are observed in body fluids of local inflammatory sites, in synovial fluid of patients with rheumatoid arthritis (100 μg/ml) and in fluid from human abdominal abscess [6,8,9].

Evidence unraveling the extracellular functions of calprotectin and its subunits is accumulating recently. For example, antimicrobial activity [11-14], and regulatory activities towards migration or adherence of inflammatory cells have been proposed [15,16].

It was found that this protein exerts a cytotoxic effect on a very broad range of tumor cell lines [17,18] and that it has an apoptotic activity in tumor cells [19]. Thus, calprotectin is recognized as one of the effective molecules in PMNs (polymorphonuclears) against tumor cells. Calprotectin has been reported to induce apoptosis in normal fibroblast cells, which occurs in the inflammatory tissues [20]. To benefit from calprotectin induction of apoptosis in treatment of tumors, it is important to understand the mechanism underlying the induced cell death. Considering the finding that calprotectin increases in colorectal cancer [21,22], this study was performed to investigate the effect of this protein complex on the colon cancer using HT29/219 (colon carcinoma) and SW742 (colon adenocarcinoma) cell lines.

Materials and Methods

Chemicals, culture media and related compounds were purchased from Sigma Co. (USA). Cell culture plasticware obtained from Nunc Co. (Denmark) and caspase-3 colorimetric assay kit (Cat. No. 101K4019) from Sigma (Germany). Caspase-9 colorimetric assay kit (Cat. No. BF10100) and annexin V- FITC apoptosis detection kit (Cat. No. TA4638) were from R&D systems Co. (USA), FITC-labeled anticalprotectin (mouse IgG$_1$ clone 27E10) (BACHEM, USA).

Cell Culture

HT29/219 (NCBI C154) human colon carcinoma, SW742 (NCBI C146) human colon adenocarcinoma cells, MOLT-4 (NCBI C149) human acute T-lymphoblastic leukemia and HL-60 (NCBI C217) human promyelocytic leukemia (obtained from National Cell Bank of Iran (NCBI)) were grown in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml

penicillin and 100 μg/ml streptomycin. They were incubated at 37°C in a humidified incubator with 5% CO_2 and 95% air. Cultures were regularly examined using inverted microscope (Micros, Austria).

MTT Assay

To evaluate the cytotoxicity effect of human calprotectin on these cell lines, MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide) colorimetric assay was performed [18]. Briefly, asynchronously growing cells (1.5×10^4 cells/ml) were transferred into a 96-well culture plates containing 200 μl of medium and incubated for 24 h. Calprotectin, at 50, 80, 100, 120, 150, 175 μg/ml were added and incubated for 12, 24, 30, 36, 48, 60 and 72 h after which MTT assay was performed. The percent of cell viability was calculated using the equation: (mean OD of treated cells/mean OD of control cells) × 100.

Analysis of Nuclear Morphology

Cells were plated in 8 well chamber slides and allowed to adhere. Calprotectin treated cells were fixed with methanol-acetic acid 3:1 (v/v) for 10 min after which staining was carried out with Hoechst 33258 (10 μg/ml) at 37°C in dark (10 min). Slides were then washed with PBS (pH 7.4) and examined by an epifluorescence microscope (Micros, Austria). Apoptotic cells were defined on the basis of nuclear morphology changes such as chromatin condensation and fragmentation. Annexin V/PI staining was carried out according to the kit procedure. Early apoptotic cells show green fluorescence.

Caspase-3, -8, and -9 Activation Assays

A caspase-3 (using DEVD-pNA as substrate), caspase-8 (using Ac-IETD-pNA as substrate) and caspase-9 (using LEHD-pNA as substrate) colorimetric assay kits were used to investigate the activation of these caspases in the treated HT29/219 and SW742 cells. Briefly, to estimate caspases-3 and -8 activity, cells were lysed by incubation with cell lysis buffer on ice for 15 minutes and then centrifuged at 20,000 g for 10 min (at 4°C). For caspase-9 activation assay, cells were lysed by incubation with cell lysis buffer on ice for 10 min and then centrifuged at 10,000 g for 1 min (at 4°C). Enzymatic reactions were carried out in a 96 well flat bottom microplate. To each reaction samples 5, 25 and 50 μl of cell lysate (100-200 μg total protein) were added for caspase-3, -8, and -9, respectively. Additional controls, one free cell lysate and the other free substrate

as well as caspase-3 and 8 positive controls have been used. Protein content was estimated by Bradford method [23]. The activities were expressed as nmole/min/mg protein.

Calprotectin Binding Assay by Flow Cytometry

MOLT-4 (positive control), HL-60 (negative control), HT29/219 and SW742 cells were washed three times with phosphate buffer saline containing 0.3% bovine serum albumin (BSA) and 0.05% sodium azide (NaN_3) (B-PBS). A total of 2×10^6 cells was incubated with 10 μg of human calprotectin for 1 h and then washed three times with B-PBS. The cells were then incubated with 200 μl of the FITC-labeled anticalprotectin antibody (1/50) (mouse IgG_1 clone 27E10) containing 20 μg/ml propidium iodide, in order to gate out dead cells, for 30 min in dark and then washed three times with B-PBS. In order to determine nonspecific binding of FITC-labeled anticalprotectin, the cells were incubated with FITC-labeled antical-protectin antibody in the absence of human calprotectin. The stained cells were analyzed on a Coulter flow cytometer (USA) using 488 nm excitation. A 496 nm bandpass filter for fluorescein detection, and a filter >600 nm for propidium iodide detection were used. Automated analyses were performed using a software. In experiments considering Ca^{+2} and Zn^{+2} on calprotectin binding to its binding site, all washing steps were performed by B-PBS containing 1 mM of each ion.

Statistical Analysis

The results were expressed as the mean ± SD and statistical differences were evaluated by one way ANOVA. P<0.05 was considered as significant.

Results

Cytotoxicity Assay

To determine cytotoxicity of calprotectin, viability test was applied using MTT assay. As it is shown in Figure 1, treatment of the HT29/219 cells with human calprotectin resulted in a significant cell death at concentrations higher than 80 μg/ml at 12 h. By increasing in the incubation time (24, 30, 36, 48, 60, and 72 h) the effective dose response decreased to 50 μg/ml (p<0.05) (Fig. 1A). Calprotectin also induced a significant cell death in the SW742 cells at all examined concentrations during the scheduled time periods (Fig. 1B).

Detection of Apoptosis Using Annexin V/PI Staining and Hoechst 33258

Fluorescein-conjugated annexin V (FL1-H) and PI (FL2-H) staining (detected by epifluorescence microscope) were used to distinguish apoptotic cells. The results showed that the cell death induced by human calprotectin in both cell lines were mainly apoptotic type (Fig. 2A and Fig. 2B). Furthermore, the apoptotic changes in the nuclear morphology including chromatin condensation and fragmentation were also observed using Hoechst 33258 staining (Fig. 3A and Fig. 3B).

Caspase 3, -8, and -9 Activation

To explore the possible biochemical mechanisms related to human calprotectin apoptosis induction, the activation of caspase-3, -8 and caspase-9 were studied. The results revealed that the activity of caspase-3 and -9 were significantly (p<0.05) increased in both cell lines treated with human calprotectin. It was observed, however, a slight increase in the activity of caspase-8 in both cell lines treated with calprotectin only at concentration above 120 μg/ml (Figs. 4 and 5).

Calprotectin Binding Site

To investigate the possible existence of calprotectin binding sites on the SW742 and HT29/219 cells, the fluorescence intensity of FITC-labeled anticalprotectin stained cells were compared with that of MOLT-4 and HL-60 cell lines as positive and negative control respectively. There was not any significant difference between non-specific binding and specific binding in HT29/219 and SW742 cell lines (Fig. 6 A and B). No significant differences were observed between these cell lines and negative control cell line (HL-60), ruling out the presence of any binding sites for human calprotectin on these cell lines (Fig. 7).

Discussion

There are few reports indicating that rat calprotectin or human recombinant calprotectin induces apoptosis in some human or mouse cell lines [17-20,24,25]. In the present study we showed, for the first time, that human calprotectin induces apoptosis in both HT29/219 and SW742 colon adenocarcinoma cell lines. The effective concentrations of calprotectin determined in this study is similar to those previously reported for rat and recombinant calprotectin (more than 100 μg/ml) [17-20,24,25].
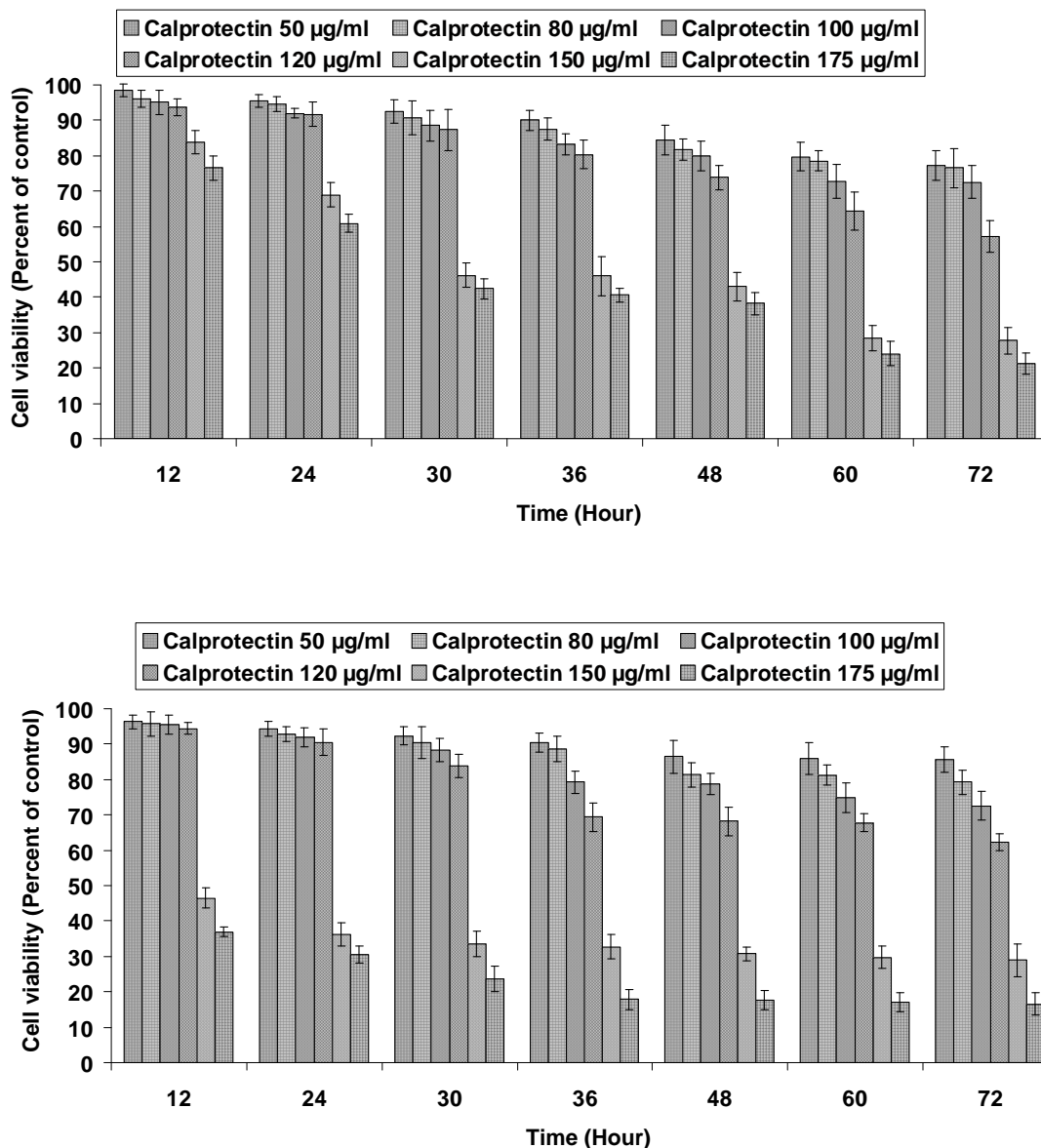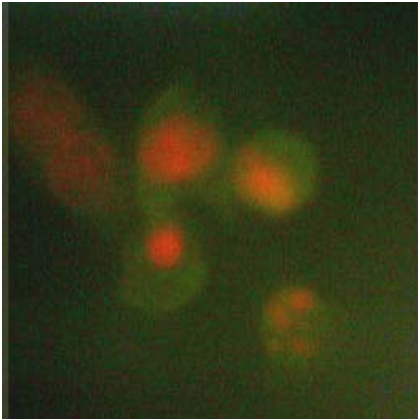
Figure 1. Effect of calprotectin on the growth of HT29/219 (A) and SW742 (B) cell lines. The cells were treated with different concentrations of calprotectin for 12 to 72 h and the viability was assessed by MTT assay. Results are expressed as percent of corresponding control and represent the mean ± SD of 4 repeats.

There are several observations supporting the idea that calprotectin induces apoptosis *via* extracellular zinc ion exclusion because the cell-death inducing activity of human recombinant, as well as rat calprotectin was completely inhibited by the co-presence of zinc ion in EL-4, mice embryonic fibroblast, and human dermal fibroblast cell line [17,18,20].

Furthermore, there might be additional factors for the cytotoxic activity exerted by calprotectin on the target cells.

It is conceivable that membrane-bound calprotectin is more effective in inducing apoptosis than free soluble protein. One plausible explanation for this would be the effective localization of calprotectin in the cells requiring zinc-chelating activity. The other explanation would be the activation of signaling pathway through which calprotectin-receptor complex may act. The presence of such binding sites has been reported in human leukemia [26,27] and endothelial cell lines [28]. Therefore the possible occurrence of these surface
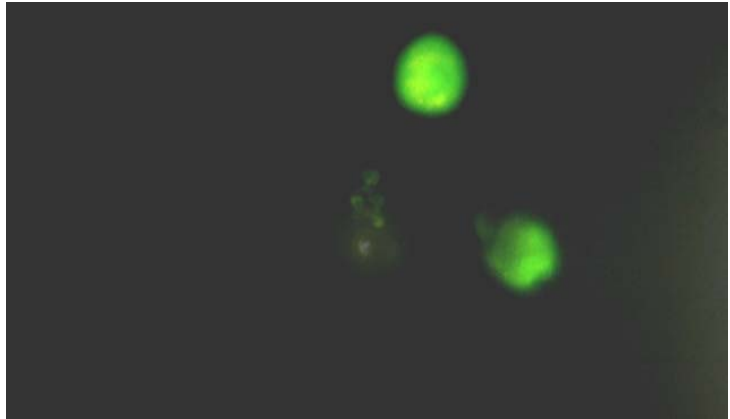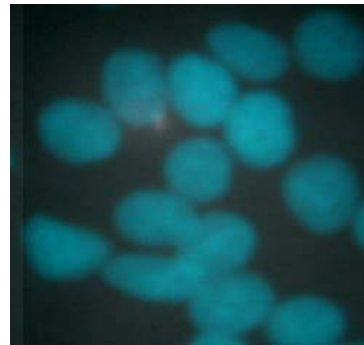
A)

B)

Figure 2. Identification of apoptotic cells by Annexin V/PI staining in HT29/219 (A) and SW742 (B) cell lines following treatment with calprotectin.
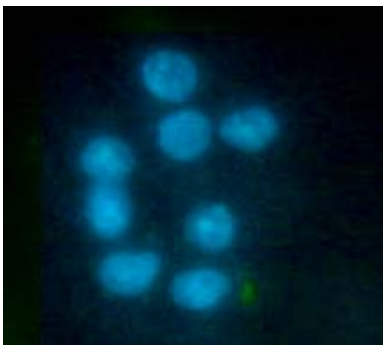
A)

         Treat   **Contol**

B)

         Treat   **Contol**

Figure 3. Nuclear morphology of HT29/219 (A) and SW742 (B) by staining with Hoechst 33258.
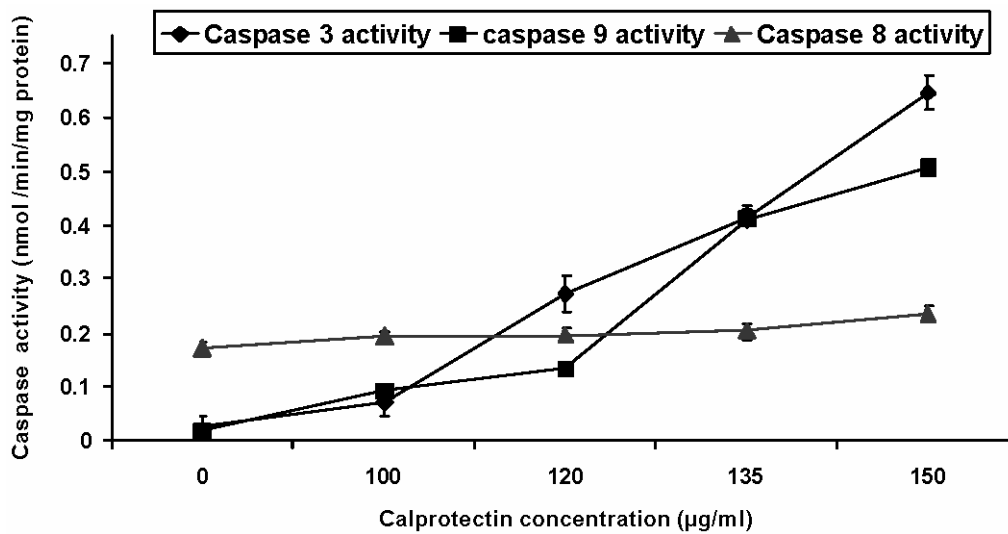
Figure 4. Activity of caspase-3, -8, and -9 in HT29/219 cell line after treatment with human calprotectin for 36 h. Results are expressed as activity of the enzyme and represent the mean ± SD of 4 repeats.

Figure 5. Activity of caspase-3, -8, and -9 in SW742 cell line after treatment with human calprotectin for 36 h. Results are expressed as activity of the enzyme and represent the mean ± SD of 4 repeats.
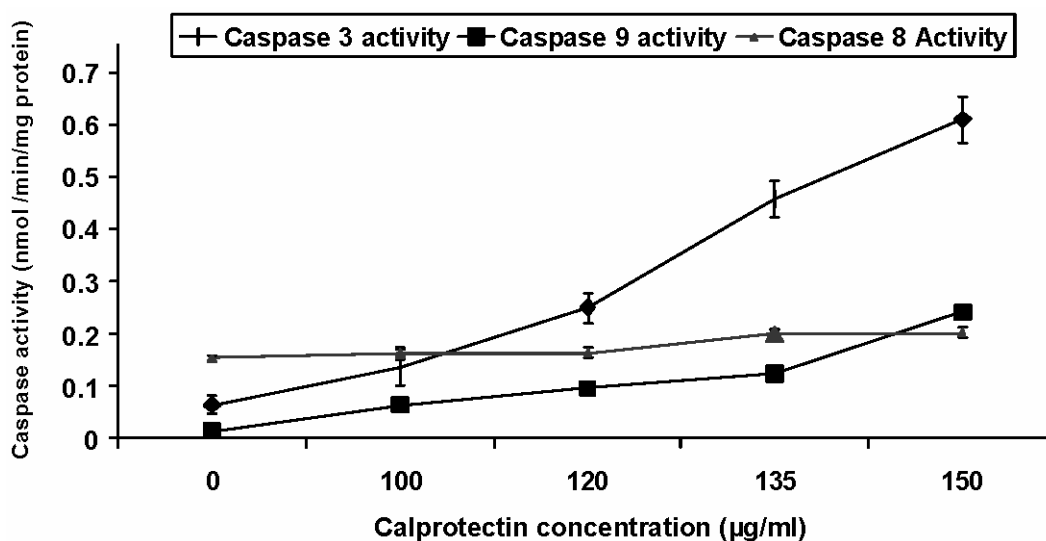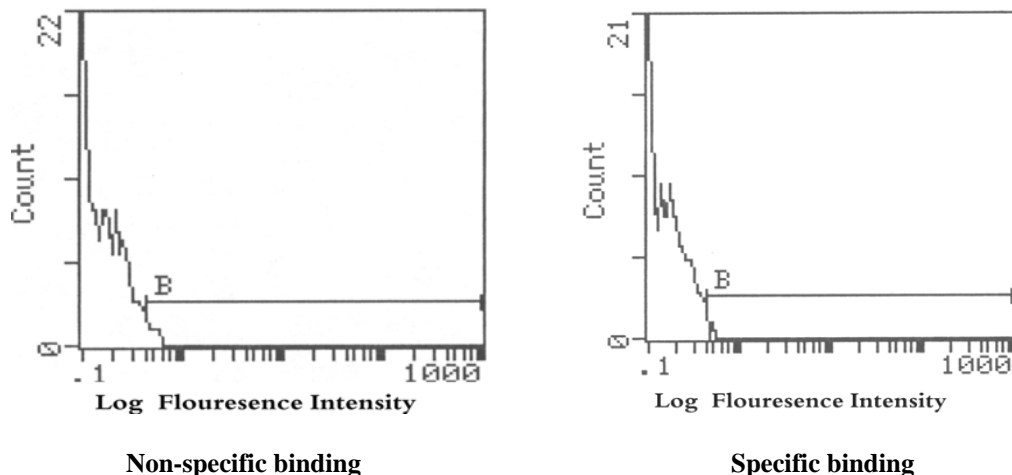
biding sites for calprotectin on the SW742 or HT29/219 cells were investigated. The results clearly ruled out the possibility of calprotectin binding to the cell membrane of these cell lines (Fig. 6C).

Activation of caspases is generally considered to be a requisite event during apoptosis. Our results also demonstrated that apoptosis induced by human calprotectin was depended on the activation of caspase-9 in both cell lines (Fig. 5A and B) suggesting a possible mitochondrial apoptotic pathway. The mitochondrion is the major role player in induction, regulation and execution of apoptosis. Mitochondria coordinate apoptosis by channeling the input pathways onto a central pathway which is governed by
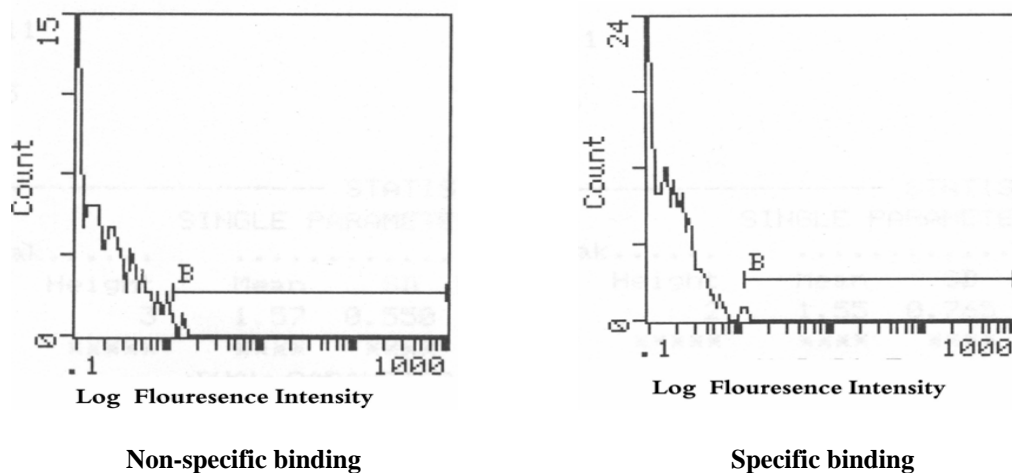
A)

Non-specific binding   **Specific binding**

B)

Non-specific binding   **Specific binding**

Figure 6. Calprotectin binding assay by flow cytometry using FITC-labeled anticalprotectin antibody (mouse IgG_1 clone 27E10) in HT29/219 (A) and SW742 (B) cell lines. There are not any significant changes between non-specific and specific bindings.

mitochondrial associated anti-apoptotic (*e.g.* Bcl-2) and pro-apoptotic (*e.g.* Bax) families of regulators and by providing a scaffolding for the proteolytic events that trigger processing and activating various members of the caspase enzyme family [29,30]. As it was reported previously, apoptotic activity of calprotectin is depended on the reactive oxygen species (ROS) in some cell lines [25]. Thus, due increment activation of caspase-9 observed in both cell lines upon treatment with calprotectin, a mitochondrial damage resulted from

ROS effect could be postulated.

Concerning caspase-3 activation (Fig. 4A and B), both treated cell lines showed a significant elevation as well. As pro-caspase-3 is stabilized in the presence of zinc ion [30,31], a decrease in the intracellular pool following extracellular zinc chelation by calprotectin might be responsible for the caspase-3 activation. Future studies can reveal how extracellular zinc chelation by human calprotectin affects the intracellular zinc concentration.
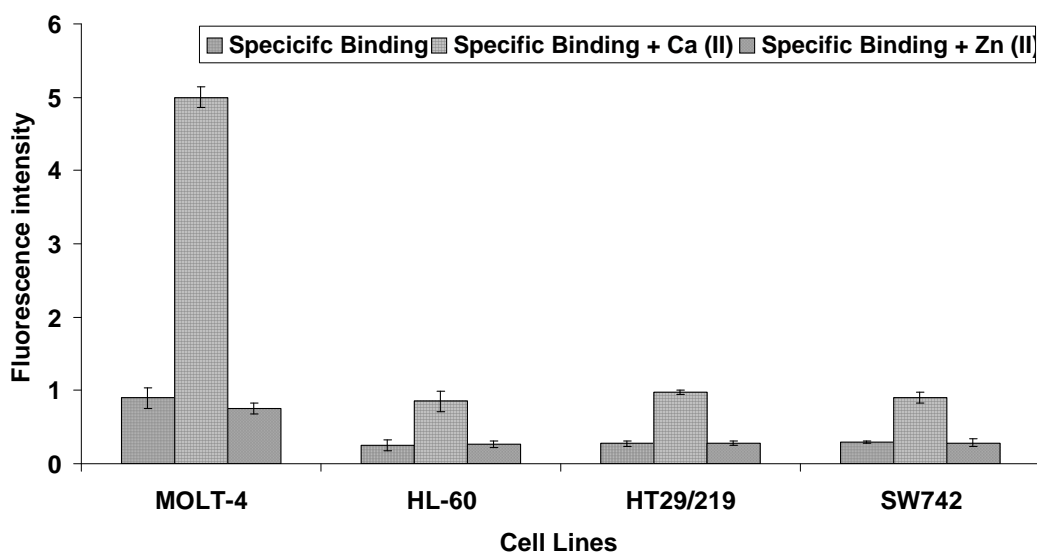
Figure 7. Comparison of calprotectin binding sites on the cell surface of MOLT-4 (positive control), HL-60 (negative control), HT29/219 and SW742 cell lines. Results are expressed as percent of cells that have calprotectin binding and represent the mean ± SD of 4 repeats.

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