Induction of apoptosis in human tumor cell lines by platelets

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Abstract

Introduction: It has been reported that platelets can eradicate tumor cells in vitro, although the mechanism of this effect has not been determined. The effect of platelets on the induction of apoptosis in tumor cells is largely unknown.
Materials and methods: To investigate this effect, two human hematologic cell lines, K562 and Daudi, were independently faced with unstimulated and thrombin-activated platelets. After the elapsed co-culture time, the levels of caspase-3 and CD95 were evaluated as a sign of cell death and apoptosis. In addition, immortalized cells were evaluated using trypan blue, 7-AAD and WST-1 methods.
Results: CD95 and caspase-3 levels were significantly increased in both cell lines compared with the control cells ($P<0.05$). Beside, considerably lower number of living cells were shown by trypan blue, 7-AAD and WST-1 in the treatment groups compared with the control group ($P<0.05$).
Conclusion: This study demonstrated the role of apoptosis in tumor inhibition and implied the ability of platelets to induce apoptosis in tumor cell lines.

Keywords: Apoptosis, Platelet, K562, Daudi, CD95

Introduction

Platelets are blood cells that play the key role in hemostasis (1). They contain a large number of biologically active proteins in the cytoplasmic granules containing alpha and dense granules, primary lysosomes and peroxisomes (2). Platelets also have several immunologically important cytokines and chemokines that are released at the time of activation (3, 4). Alpha granules contain a number of important growth factors, including vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and transforming growth factor beta (TGFβ) that are effective in improving wounds and angiogenesis (5) Platelet-derived VEGF not only facilitate the angiogenesis but also increase vascular permeability and the further spread of tumor cells (6). Beside, in addition to the effective factors of angiogenesis, platelets contain antiangiogenic factors such as PF4, TSP-1 and endostatin that can limit the growth and survival of cancer cells (7). This could reveal different roles of platelet secretory factors in pathophysiological process in the body. The anti-metastatic factor; PF4 can be as an available candidate in cancer therapy (8). Further investigations have revealed that the majority of tumor cells or the primary tumor tissues are able to produce soluble factors such as ADP and thrombin that could cause platelet activation as well as platelet or platelet-tumor aggregations (9-10). Activated platelets and microparticles released from platelets included the functional adhesion receptors of P-selectin and CD40L (11, 12). At the time of

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activation, platelets release two types of vesicles containing platelet microparticles and exosomes. Under the physiological conditions, these membrane vesicles are responsible for transmitting signals of platelets to other cells. Platelet-derived microparticles sprout from the membrane of activated platelets and have the membrane molecules of platelets such as CD40L that can react with cells containing CD40 receptor (13). The role of platelets in immune and inflammatory responses and defense against pathogens and malignancies is primarily occurring through several molecules, including TLR1, MHC-1, CD40 and CD40L (14). Platelets have different factors of cell growth induction or cell growth inhibition. After the co-culture of platelets with the neoplasm cells, platelets impose the cell growth inhibition effects using different mechanisms by cytotoxic effects (15, 16) or cell cycle preventing (17). Because of the lack of information in the field of apoptosis induction of platelets on tumor cells, we studied this issue using treatment of two cell lines with platelets or thrombin-activated platelets.

Materials and methods

Preparation of platelets: Five single donor platelet concentrate bags (JMS Singapore Plte Ltd. contained CPDA-1 solution) were prepared from Iranian Blood Transfusion Organization (IBTO). Informed consent was obtained from the blood candidates by (IBTO). Platelets were centrifuged at 300g for 5 min to remove red blood cells (RBCs) and white blood cells (WBCs). The supernatant was centrifuged at 1200 g for 10 min to sediment platelets. Platelets were counted by an automated hematology analyzer (Sysmex K-1000, Kobe, Japan).Additionally; platelets were activated in the presence of thrombin (3U/ml) at room temperature.

The cell lines: Two tumor cell lines were used in the present study, K562 (human chronic myelogenous leukemia cell line) and Daudi (human B Lymphoblast cell line) were provided by (NCBI) national cell bank of Iran, Pasteur Institute (Tehran, Iran).

The co-culture of platelets and cell lines: Unstimulated and thrombin-activated platelets (5×10^7cells), were independently introduced into the culture medium included K562/ Daudi (1×10^5 cells) in the wells of a cell culture plate. The co-culture medium consisted of RPMI 1640 supplemented with 10% FBS, 1% penicillin and streptomycin (GIBCO, USA), prepared from a stock solution of 100 X concentration of 10,000 IU/mL penicillin and 10,000 µg/mL of streptomycin and 1% L-glutamine (200 mM). The culture plates were incubated at 37ºC for 48 hours in a humidified incubator with 5% CO2 atmosphere. It was worthy of note that all co-cultures were performed in duplicate in the presence of appropriate controls. Controls of this study consisted of K562 or Daudi cells without any treatment.

7-AAD viability staining: 7-Aminoactinomycin D (7-AAD) is a fluorescent intercalator that undergoes a spectral shift upon association with DNA. After the co-culture time, cells in each well were harvested and centrifuged at 400g for 5 min to remove platelets. Deposited cells were washed once with assay buffer (Cayman 7-AAD assay kit, USA), resuspended in the staining solution of the kit and incubated at room temperature for 15 min at dark. Cells were centrifuged at 400 g for 5 min and suspended in assay buffer for analysis with flow cytometry technique.

CD95 expression levels: The expression of CD95 was studied on K562 and Daudi cells after the co-culture time by flow cytometry technique. In a one-step method, 2 µl of FITC-conjugated mouse anti-CD95 antibody (eBioscience, USA) was added to the tubes each included 10^5 cells in 100 µL. The tubes were left for 40 min at 4ºC before analysis by flow cytometry .

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Active caspase-3 concentration: The experiment was based on the measurement of P17 subunit of active caspase-3 inside the human cells using ELISA method (Abcam, UK). Forthy-eight hours after the co-culture, K562 and Daudi cells were lysed by the extraction buffer included in the kit after the addition of the protease inhibitor (1 mM PMSF).The extracted protein solutions were stored at -80ºC. ELISA method was done as the manufacturer's instructions.

WST-1 cell proliferation and viability assay: WST-1 is a method to measure the cell proliferation. Expansion in the number of viable cells results in an increase in the activity of the mitochondrial dehydrogenases, which in turn leads to increase in the amount of formazan dye formed. After the co-culture time, cells were centrifuged at 400 g to remove platelets. Afterwards, 10 µL of WST-1 reagent (Cayman cell proliferation assay kit, USA) was added to each well containing 100 µL of cells in a 96 well plate. Subsequently, the plate was incubated for 4 hours at 37ºC. The absorbance of wells was measured with a microplate absorbance reader at the wavelength of 450 nm.

Trypan blue exclusion test of cell viability: Trypan blue provides the possibility for detection of live and dead cells, microscopically. After the end of co-culture time, the number of viable and dead cells was enumerated for the cells of each well by trypan blue. Finally, the percent of dead cells was calculated.

Statistical analysis

The non-parametric; Wilcoxon method with SPSS 16.0 software was used to compare the results of this experiment. The $P<0.05$ was considered statistically significant.

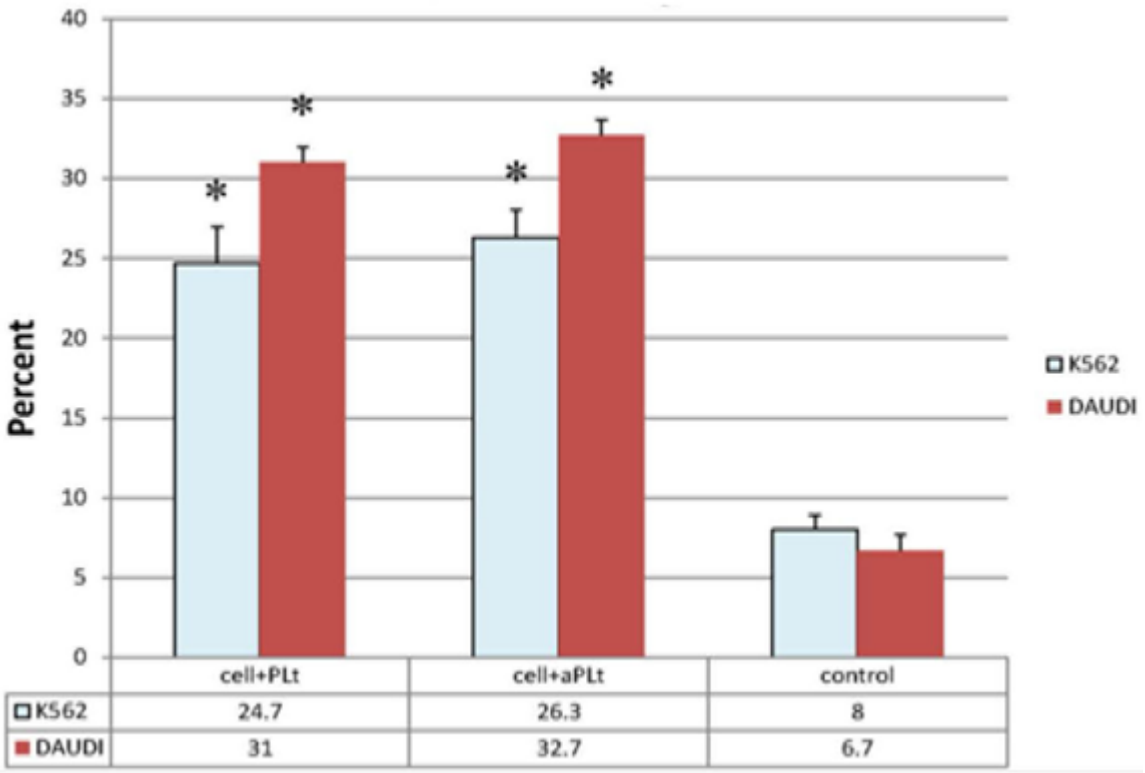


Figure 1. K562 and Daudi cell lines were treated with platelets (plt) or thrombin-activated platelets (aplt), in the 24-well culture plates. Dead cells were labeled with 7-AAD dye and measured by flow cytometry technique. Data were presented as the mean±SD of five independent experiments. * Indicates $P<0.05$.

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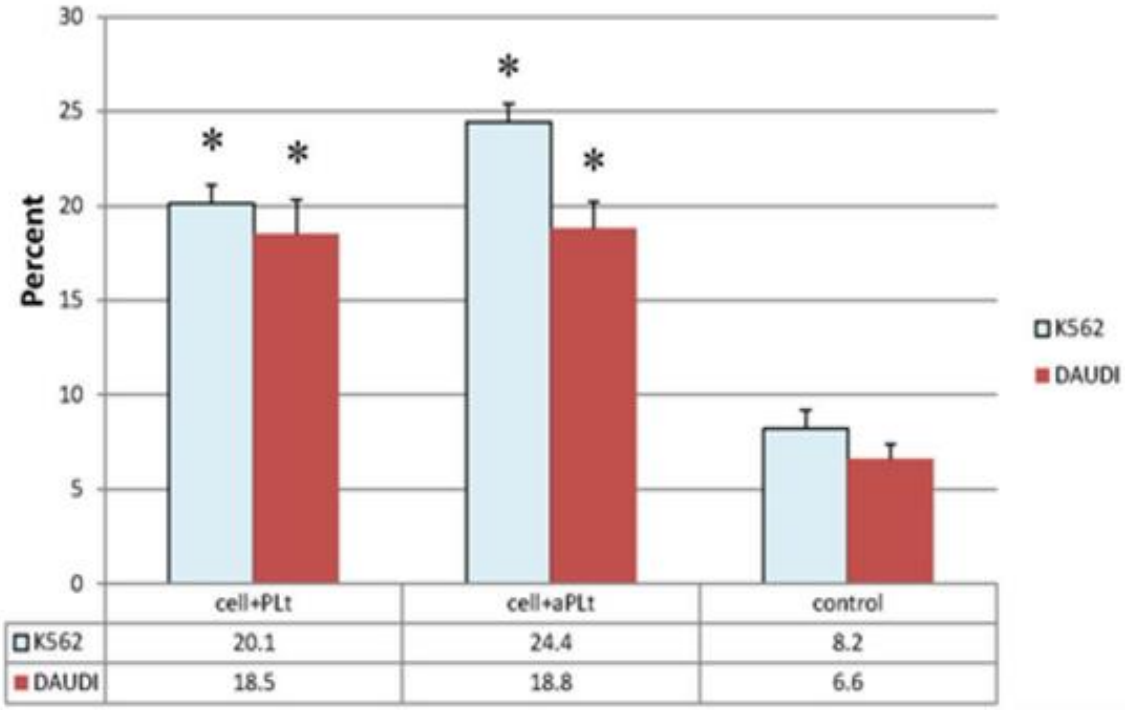
Results

7-AAD viability staining results: The percent of 7-AAD-stained cells was measured by flow cytometry technique (Figure 1) and showed significantly greater amounts in the two treatment groups than control group (P<0.05).

CD95 expression: CD95 (FAS) expression was analyzed on K562 and Daudi cells after 48 hours of co-culture using the flow cytometry technique. Significantly higher expression of CD95 was detected in the treatment groups compared with the untreated control group (P<0.05) (Figure 2).

Active caspase-3 concentration: The concentration of human active caspase-3 was significantly increased in the extracts of K562 and Daudi cells after the co-culture time compared with the control group (P<0.05) (Figure 3).

Trypan blue and WST-1 results: Trypan blue (Figure 4) and WST-1 (Figure 5) showed significantly increased number of dead cells in the cell lines after the treatments in compare with the untreated control cells. The differences between the treatment cases with control was significant (P<0.05).



	cell+PLt	cell+aPLt	control
K562	20.1	24.4	8.2
DAUDI	18.5	18.8	6.6

Figure 2. K562 and Daudi cell lines were treated with platelets (plt) or thrombin-activated platelets (aplt), in the 24-well culture plates. Higher expression of CD95 was shown in the treatment groups compared with the control group. Data were presented as the mean±SD of five independent experiments. * Indicates P<0.05.

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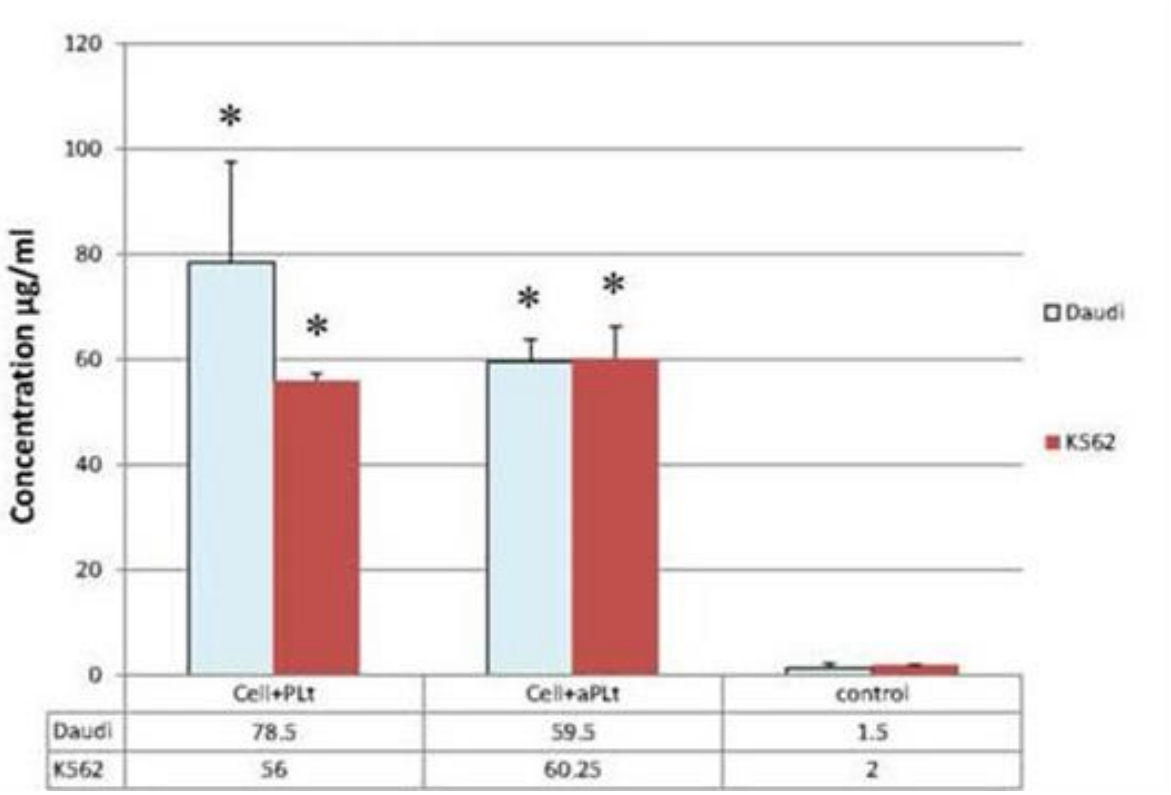


Figure 3. Active caspase-3 levels in K562 and Daudi cell lines after treatment with platelets (plt) or thrombin-activated platelets (aplt). Higher concentrations of caspase-3 were shown because of the platelets (plt) or thrombin-activated platelets (aplt). Data were presented as the mean±SD of five independent experiments. *Indicates P<0.05.

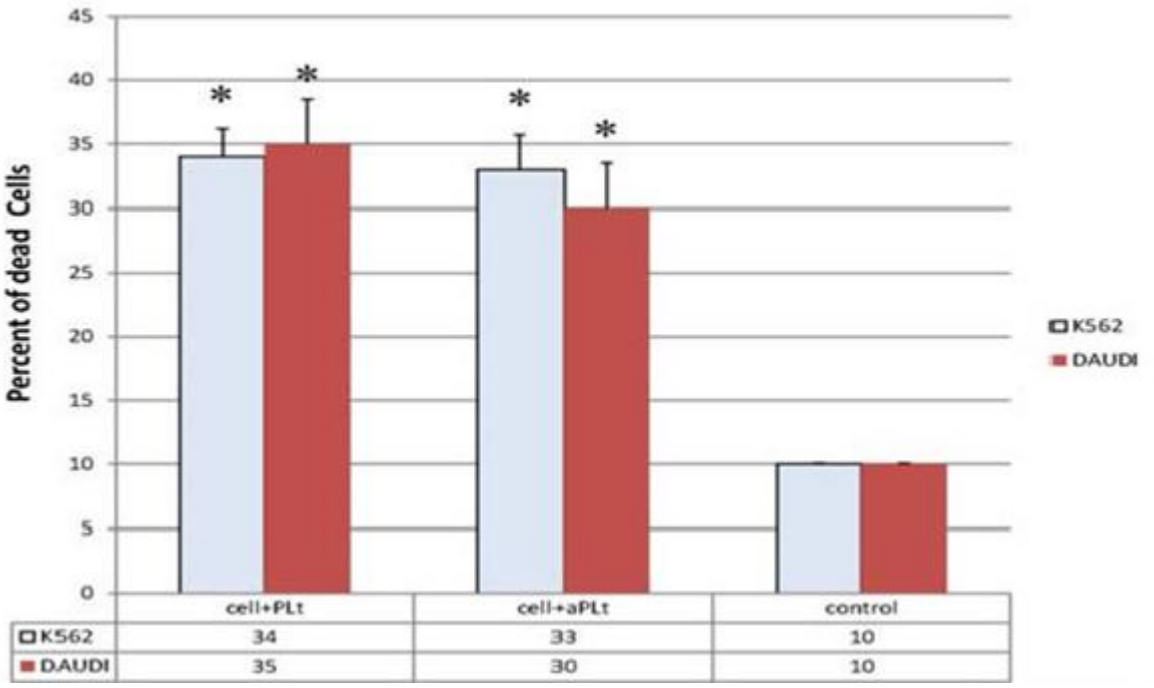


Figure 4. Trypan blue results. Plasma membrane of non-viable cells was permeable to trypan blue whereas live cells remained unstained. The number of viable and dead cells was counted microscopically after treatment with platelets (plt) or thrombin-activated platelets (aplt). The percent of dead cells was calculated. Data were presented as the mean±SD of five independent experiments. * Indicates P<0.05.

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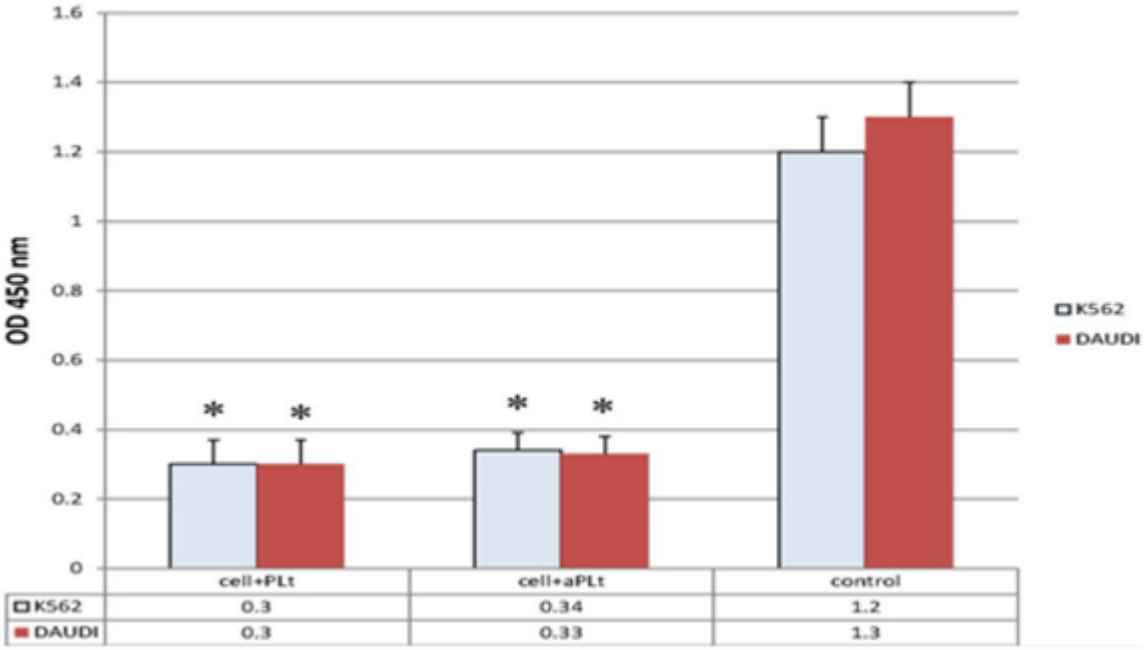


Figure 5. WST-1 results. Enzymatic cleavage of the tetrazolium salt (WST-1) to formazan by cellular mitochondrial dehydrogenases was measured with absorbance reading at the wavelength of 450 nm. Exposure with plts or thrombin-activated platelets (aplt) caused lower viability and metabolic activity of the cell lines. Data were presented as the mean±SD of five independent experiments. * Indicates P<0.05.

Discussion

Despite the role of platelets in tumor spread in the body (18), the cancer killing effects of platelets has been identified for some cell lines (15,19). We studied the viability and apoptosis of cell lines after co-culture with platelets or thrombin-activated platelets. The results indicated that both platelets and thrombin-activated platelets could cause growth inhibition and apoptosis in the K562 and Daudi cells after 48 hours of co-culture.

Our study with K562 and Daudi cells were correlated with the studies of some researchers. Examples: Kawamura and co-workers showed that platelets have roles in cell growth inhibition and killing of some neoplasm cells (20). Ible and co-workers showed that the produced materials in the arachidonate metabolism pathway of platelets were toxic for some tumor cell lines (15). Bykovskaya and co-workers demonstrated that platelets isolated from the blood of oncology patients as well as healthy people can cause cytotoxic effects on several types of tumor cell lines (21).

However, the findings of this study were disagreed with that of Wang who claimed that the inhibition of tumor cell growth by platelets was mainly due to the cell cycle arrest and not apoptosis or cell death (17).

We also showed that the thrombin-activated platelets and unstimulated platelets both have nearly similar influences on tumor cell lines. Correlated with us, Sagawa and co-workers reported that the thrombin-activated platelets such as unstimulated platelets have cytotoxic effects on some tumor cell lines but due to different morphological changes made in tumor cells, their effects may be carried out with different mechanisms (16).

The present study showed high expression levels of CD95 (FAS) on the studied cells after treatment with platelets or thrombin-activated platelets. This effect may be related to soluble or cell surface CD40L on platelets. It has been found that CD40-CD40L ligation, up regulates Fas expression on tumor cells (22, 23) and enhanced FAS expression should

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predispose cells to apoptosis (24).On the contrary, Gogstad showed that the supernatant of thrombin-activated platelets had no cytotoxic effects on tumor cells although it contained various soluble materials that are secreted from activated platelets (25).

The results of this study showed similarity between the effects of platelets and thrombin-activated platelets on cancer cells. We described the apoptotic cell death effects of unstimulated platelets and thrombin-activated platelets on K562 and Daudi cells for the first time. This study implied the capability of platelets or plt-derived components in growth inhibition of some tumor cells under the appropriate conditions. Further studies are needed to clear the effective factors of platelets in tumor growth inhibition.

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