

# Relationship between activation and apoptosis in platelets

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## ABSTRACT

Phosphatidylserine (PS) externalization is a marker for the nucleated cell apoptosis, and refers cellular activation rather than apoptosis in platelets. On the other hand, several similarities exist between platelet activation and apoptosis in nucleated cells. Herewith we investigated the relationship between platelet activation and platelet apoptosis. Platelets isolated from fresh blood of 22 healthy volunteers were incubated with and without calcium ionophore A23187. Platelet activation was evaluated with CD62P and CD63 antibodies, whereas apoptosis with intracellular anti caspase 3-antibody and JC-1 cationic dye. In order to detect PS externalization we used Annexin V by flow cytometry at the beginning, 20<sup>th</sup> minute and 5<sup>th</sup> hours of the incubation, respectively. There were positive correlations between caspase-3 activation and PS externalization,  $\Delta\psi_m$  depolarization, CD63, and also between PS externalization and CD62P in incubations with A23187 at 5<sup>th</sup> hours of incubations. These results suggest that there is a relationship between activation and apoptosis in platelets, and platelet activation may progress to platelet apoptosis.

**Key Words:** Annexin V, apoptosis, phosphatidylserine, platelet activation

## ÖZET

### Trombosit aktivasyonu ve apoptozu arasındaki ilişki

Çekirdekli hücrelerde fosfatidilseri (PS)'nin hücre zarı dış yüzeyine çıkması bir apoptoz belirteci olup, trombositlerde apoptozdan ziyade aktivasyonu düşündürmektedir. Diğer taraftan, çekirdekli hücrelerdeki apoptoz ve trombositlerdeki aktivasyon arasında birçok benzerlik bulunmaktadır. Bu çalışmada trombosit aktivasyonu ve apoptozu arasındaki ilişkiyi araştırdık. Sağlıklı 22 vericiden taze olarak alınan kandan izole edilen trombositler calcium ionophore A23187 bulunan ve bulunmayan tüplerde inkübe edildi. Trombosit aktivasyon belirteci olarak CD62P ve CD63, apoptozunun belirteci olarak anti-caspase-3 antikoru ve JC-1 katyonik boyası, hü-

re dış yüzeyindeki PS tespiti için ise Annexin-V kullanıldı. Tüm belirteçler A23187 ile inkübasyon başlangıcında, 20. dakikada ve beşinci saatte akım sitometrisi ile çalışıldı. Inkübasyonun beşinci saatinde caspase 3 aktivasyonu ile PS eksternalizasyonu,  $\Delta\psi_m$  depolarizasyonu, CD63 arasında ve ayrıca PS eksternalizasyonu ile CD62P arasında anlamlı korelasyon bulundu. Bu bulgular trombosit aktivasyonu ve apoptozu arasında bir ilişkinin olduğunu, trombosit aktivasyonunun apoptozu ile ilişkisini düşündürmektedir.

**Anahtar Kelimeler:** Anneksin V, apoptoz, fosfatidilserin, trombosit aktivasyonu

## INTRODUCTION

Phosphatidylserine (PS) is one of the major phospholipids distributed asymmetrically in the bilayer of the cell plasma membrane and is normally located to the membrane's inner leaflet<sup>[1,2]</sup>. Transbilayer migration of PS to the outer leaflet may result in macrophage stimulation and acceleration of thrombin generation in platelets<sup>[3,4]</sup>. It is a marker of apoptosis in nucleated cells<sup>[5]</sup>. There are many similarities between nucleated cell apoptosis and platelet activation, such as PS externalization. Mitochondria membrane potential deprivation ( $\Delta\psi_m$  depolarization), caspase activation and nuclear fragmentation are the other major events of nucleated cell apoptosis<sup>[6-8]</sup>. Microparticle formation in activated platelets resembles membrane blebbing in apoptotic nucleated cells<sup>[8]</sup>.

There is no satisfactory information in the literature about the consequences of the activated platelets. Similarities between platelet activation and nucleated cell apoptosis suggest that there may be a link between them. In this study, we investigated the role of PS externalization in platelet activation and platelet apoptosis.

## MATERIALS and METHODS

### Materials

Calcium ionophore A23187 was obtained from Sigma (St Louis, USA) and CD41-FITC and CD62P-FITC were obtained from Dako (Glostrup, Denmark). J-aggregate-forming lipophilic cationic fluorochrome 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1, Mito PT) was obtained from Immunochemistry Technologies (MN, USA). Anti-active caspase 3-PE and Annexin

V-PE (Ann V-PE) were purchased from BD Biosciences (CA, USA). CD63-FITC was obtained from Ancell Corporation (MN, USA).

### Platelet Isolation

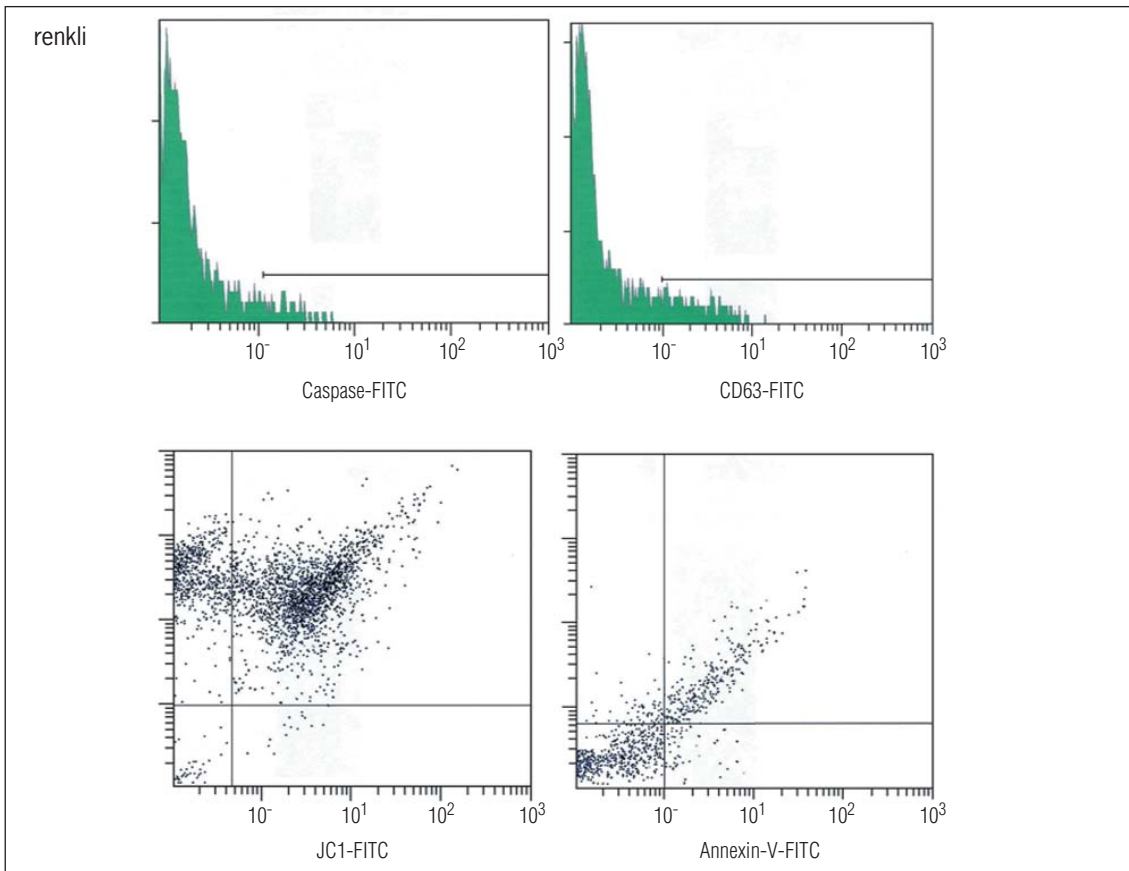
Platelets were prepared using fresh blood samples of healthy volunteers (20-30 years old) who had not received any medications in the last 10 days. A 21-gauge needle attached to a 5 mL syringe was used for blood donation. The blood sample was transferred into vacuum-sealed tubes (BD Diagnostics) containing sodium citrate. The tubes were centrifuged at 100 x g for 10 minutes in a centrifuge (Nüve, NF80R). The platelet rich plasma (PRP) supernatant was transferred to another tube. More than 99% of cells in PRP, measured by automated cell counter (Beckman Coulter, LH750), were platelets. These tubes were centrifuged at 1000 x g for 10 minutes to obtain platelet poor plasma (PPP).

### Platelet Incubation

Each platelet sample was diluted with PPP in order to obtain two new tubes, each containing  $1 \times 10^6$  cells/mL. We added 10  $\mu$ mol/L calcium ionophore A23187 to one of the tubes, and kept the remaining one as control sample. Both tubes were incubated at room temperature for five hours.

### Flow Cytometric Analysis

Flow cytometric analysis was performed in the beginning for baseline value, at the 20<sup>th</sup> minute for activation and at the 5<sup>th</sup> hour of incubation for apoptosis. CD 62P and Ann V were tested together in a single tube, while CD 63, JC-1 and Caspase 3 were each tested in separate tubes (Figure 1).



**Figure 1.** Samples of the flow cytometric analyses.

Each tube, containing  $1 \times 10^5$  cell/mL, was centrifuged at  $1000 \times g$  for 10 minutes at  $22^\circ\text{C}$ . The supernatants were discharged and then washed with PBS. After washing, platelets were diluted into 100  $\mu\text{L}$  of buffer solution, and 5  $\mu\text{L}$  Ann V-PE and 10  $\mu\text{L}$  CD62P-FITC were added as instructed by the producer of apoptosis. Cell suspension was incubated in the dark at room temperature for 20 minutes, and then 400  $\mu\text{L}$  of buffer solution was added before flow cytometric analysis.

For CD41 and CD63,  $1 \times 10^5$  platelets in 50  $\mu\text{L}$  PBS and 10  $\mu\text{L}$  fluorescein conjugated antibody were incubated in the dark at room temperature for 20 minutes, in different tubes. Samples were suspended in 1 mL PBS before immediate analysis.

$\Delta\psi_m$  changes were determined with JC-1. Use of the MitoPT kit allows the easy distinc-

tion between non-apoptotic red fluorescent cells and apoptotic green fluorescent cells. JC-1 was dissolved in 500  $\mu\text{L}$  DMSO for stock solution and stored at  $-20^\circ\text{C}$  in separated tubes. The stock solution was further diluted in platelet buffer on the day of staining. Platelets ( $1 \times 10^5$ ) were diluted in 500  $\mu\text{L}$  buffer and incubated with 5  $\mu\text{L}$  JC-1 for 15 minutes in the dark at  $37^\circ\text{C}$ . 1.5 mL platelet buffer was added and centrifuged at  $1000 \times g$  for five minutes and supernatant was discharged. One mL buffer was added and centrifuged at  $1000 \times g$  and supernatant was removed. One mL buffer was added and samples were analyzed immediately with a flow cytometer.

For intracellular active caspase three staining, platelets were washed twice with cold PBS, then re-suspended in cytofix/cytoperm solution at a concentration of  $1 \times 10^5$  cells/0.5

mL. Cells were incubated for 20 minutes on ice and washed twice with perm/wash buffer at a volume of 0.5 mL. Washed platelets with perm/wash buffer were re-suspended in 100 µL perm/wash buffer and 20 µL antibody was added and incubated for 30 minutes in the dark at room temperature. Cells were washed with 1 mL perm/wash buffer, and then suspended in 0.5 mL perm/wash buffer before immediate analysis.

Samples were analyzed on the flow cytometer (Beckman Coulter, LH750), equipped with a 488-nm wavelength argon laser. The fluorescence of FITC and PE was detected by using 530 + 20-nm and 585 + 20-nm band pass filters, respectively. Platelet derived microparticle population was identified by both light scatter and CD41-FITC fluorescence. Flow cytometric analysis was performed in microparticle free area, and 10.000 platelets were analyzed for each marker.

**Statistical Analysis**

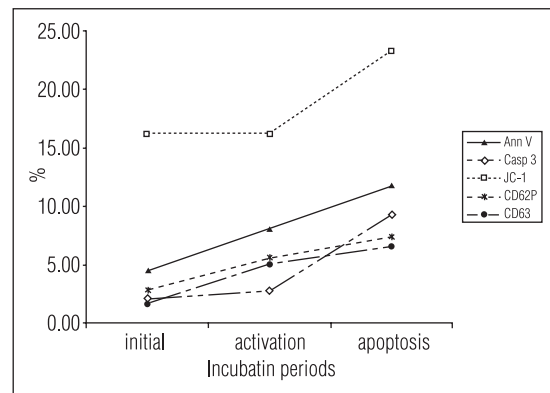
Statistical analyses of the results were performed with SPSS, 10.0. Data are presented as mean ± standard deviation (SD). Pearson's correlation test and paired-t test were used.

**RESULTS**

Results of the flow cytometric analysis at the beginning, 20<sup>th</sup> minute and 5<sup>th</sup> hour of incubation with calcium ionophore A23187 are shown in Table 1.

There was a significant difference between initial and activation periods in CD62P, CD63 and Ann V. A significant difference was also obtained between activation and apoptosis periods in Ann V, active caspase 3 and Δψ<sub>m</sub> depolarization (Figure 2). There was no significant difference between any parameters in the control samples at the beginning, 20<sup>th</sup> minute and 5<sup>th</sup> hour of incubation without calcium ionophore.

There was no correlation between Ann V, CD63, CD62P, active caspase 3 and Δψ<sub>m</sub> at the 20<sup>th</sup> minute of incubation. There were significant correlations between active caspase 3 and Ann V, Δψ<sub>m</sub> depolarization, and CD63 at the 5<sup>th</sup> hour of incubation. A significant correlation was also observed between Ann V and CD62P at this period.



**Figure 2.** Relationship between Ann V and other markers at 20<sup>th</sup> minutes and 5<sup>th</sup> hours of incubation with calcium ionophore A23187.

**Table 1.** Results of the flow cytometric analyses.

Marker	Analysis time					
	Initial* (SD)	P	Activation** (SD)	P	Apoptosis*** (SD)	
Ann V	4.50 ± 3.13	0.010	8.10 ± 4.08	0.036	11.81 ± 6.97	
Casp 3	2.10 ± 1.11	0.089	2.77 ± 1.50	0.001	9.26 ± 6.54	
JC-1	16.19 ± 8.01	0.958	16.05 ± 8.33	0.043	23.52 ± 15.97	
CD62P	2.86 ± 1.56	0.039	5.47 ± 4.88	0.256	7.36 ± 4.83	
CD63	1.67 ± 1.17	0.046	5.01 ± 7.21	0.427	6.64 ± 5.38	

SD: Standard deviation,

\* Beginning of the incubation,

\*\* 20<sup>th</sup> minutes of the incubation,

\*\*\* 5<sup>th</sup> hours of the incubation.

## DISCUSSION

Apoptosis is a complex process affecting multiple cellular components, including plasma membrane, mitochondria, caspases and DNA. Major markers of apoptosis in nucleated cells are PS externalization,  $\Delta\psi_m$  depolarization, caspase activation, nuclear fragmentation and characteristic morphologic appearance, cell shrinkage and membrane blebbing.

Apoptosis of platelets, which are enucleated cells, is still not well understood. In addition, there are many similarities between platelet activation and platelet apoptosis. As a result, there are difficulties in the description of apoptosis in platelets, and it is sometimes controversial. Theoretically, apoptosis of enucleated cells involves all footsteps of the apoptotic nucleated cell, excluding nuclear fragmentation.

Transbilayer migration of PS to the outer plasma membrane leaflet is a major event of nucleated cell apoptosis, and additionally serves as an acceleration of thrombin generation in platelets<sup>[3,4]</sup>. Similar to nucleated cells undergoing apoptosis, activated platelets express PS on the outer leaflet of the plasma membrane<sup>[8-12]</sup>.

We used CD62P and CD63 for detection of platelet activation, and active caspase 3 and JC-1 ( $\Delta\psi_m$  depolarization) for detection of platelet apoptosis, as also used by Leytin et al.<sup>[13]</sup>. CD62P and CD63, activation markers, increased significantly beyond the activation period but were not significantly increased later, in the apoptosis period. Although Ann V increased significantly in both periods, a positive correlation between Ann V and CD62P was observed only in the apoptosis period.

Ann V, reflecting PS externalization, gradually increased from the initial to the 5<sup>th</sup> hour of incubation. There were significant differences between initial and activation and apoptosis periods. It is suggested that PS externalization increases with activation and also continues later. Although CD62P and CD63 also increased significantly in the activation peri-

od, there was no correlation between these markers and Ann V. In contrast, a strong correlation between Ann V and CD62P expression during the apoptosis (or late activation) period suggests a relationship between activation and apoptosis of platelets. This finding suggests PS externalization might be related to apoptosis rather than activation.

The role of caspases in platelet activation is still controversial. It is reported that caspase activation is related to late platelet activation and PS externalization<sup>[7,8,14,15]</sup>. Wolf et al. reported that there was no caspase activation in the platelet activation period<sup>[11]</sup>. Interestingly, increased caspase three activity associated with apoptotic morphology has been observed in stored platelets<sup>[12,15,16]</sup>.

In this study, both the significant increase of caspase activation after the activation period and positive correlation between caspase three activation and Ann V in the apoptosis (or late activation) period suggest that PS externalization might be related to platelet apoptosis.

Even though the role of the mitochondria has not been well understood, increase in expression of pro-apoptotic Bak and Bax after stimulation of platelets by calcium ionophore A23187 is reported<sup>[1-3,11]</sup>. Leytin et al. also showed  $\Delta\psi_m$  depolarization in platelets by thrombin stimulation<sup>[17]</sup>. Increase of  $\Delta\psi_m$  depolarization and its positive correlation with caspase three activation in the apoptosis period in this study support the theory that a mitochondria-mediated pathway might be involved in platelet apoptosis.

On the other hand, apoptosis markers,  $\Delta\psi_m$  depolarization and caspase three activation increased only in the apoptosis or late activation period, but only caspase 3 activation was positively correlated with Ann V in this period.

In summary, Ann V was the only marker that was significantly increased in both periods, and was positively correlated with one activation and one apoptosis marker in the apoptosis period.

In conclusion, findings of this study suggest that there may be a link between activation and apoptosis in platelets, and thus this activation may progress to apoptosis; PS externalization may be the key event in this process.

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