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Annexin V and CXCR4 Expression in Adult Immune Thrombocytopenia. Control Randomized Study.

(Annexin in ITP)

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Abstract

Background: Annexin V is a cellular protein used to detect apoptotic cells by its ability to bind to phosphatidylserine, a marker of apoptosis when it is on the outer leaflet of the plasma membrane. CXCR4 is expressed on cells of megakaryocytic series, especially platelets, and plays an important role in megakaryopoiesis. Both were used to study the pathogenesis of ITP in children. This study aimed to evaluate Annexin V as a marker of early apoptosis and the CXCR4 expression as a marker of megakaryopoiesis in adult patients with ITP. **Patients and methods:** This study was conducted on 60 patients with ITP with a mean age of 39.23 ± 12.49 and 40 healthy subjects with a mean age 36.08 ± 9.4 as a control group. Using flow cytometry, we investigated apoptotic markers of platelets annexin V and megakaryopoiesis marker CXCR4 expression. **Results:** The percentage of the platelet with early apoptosis-positive markers (Annexin -D1) were less frequent in the ITP group compared to the control group ($p < 0.001$), the percentage of the platelet with late apoptosis-positive markers (Annexin -D2), living platelet (Annexin-D3), necrotic platelet (Annexin-D4) were more frequent in the ITP group compared to the control group ($p = 0.62$, $p < 0.001$, $p = 0.016$) respectively. The percentage of the platelets with CXCR4 expression was lower in ITP patients compared to the controls ($p < 0.001$). **Conclusion:** pathogenesis of ITP in adults is complicated. Platelet Annexin V together with CXCR4 expression may detect apoptosis resistance in adult patients with ITP.

Keywords

Immune thrombocytopenic purpura (ITP), Annexin V, Chemokine Receptor type 4 (CXCR4).

1. Introduction

Immune thrombocytopenia (ITP) is an autoimmune disorder characterized by a peripheral blood count of less than $100 \times 10^9/L$ (1). The most serious complication of thrombocytopenia is intracranial

hemorrhage (2,3). Primary ITP constitutes 80% of diagnosed patients (4), after excluding drugs, autoimmune diseases, and viral infections as the second cause of thrombocytopenia (5-7). Both increased platelet destruction and reduced production are responsible for the disease (8).

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Glycoprotein IIb /IIIa and their destruction by macrophages or cytotoxic T cells are the main pathogenic mechanism of ITP. This is aggravated by the associated megakaryocytic dysfunction and reduced thrombopoietin production (9,10). Both platelets, and megakaryocytes, have functional pathways of programmed cell death that were found to be abnormally enhanced in ITP (11-13). Externalization of phosphatidylserine (PS) on the surface of cells undergoing apoptosis is needed for recognition of unwanted self to be cleared by phagocytes, followed by loss of membrane integrity with eventually cellular apoptosis or necrosis (14,15). Increased PS exposure was reported in adult patients with chronic ITP (16). Annexin V has a high affinity for PS. Staining with Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) for different stages of apoptosis (17-19). A chemokine receptor for stromal cell-derived factor (SDF-1) is present on the surface of both megakaryocytic lineage and the platelet membrane known as Chemokine Receptor type 4 (CXCR4) (20–22). CXCR4/SDF-1 axis plays an important role in platelet production. (23,24) This axis also activates many intracellular signals including increases in Ca^{2+} influx, activation of phosphatidylinositol 3-kinase, and Akt activation (25–27). Akt activation leads to apoptosis resistance (28). So this axis contributes to the regulation of both physiological pathological processes.

This study aims to evaluate Annexin V and CXCR4 expression in adult ITP to understand the pathogenesis of ITP in adults and to study their utility as predictors of disease chronicity.

2. Subjects and methods

This study was approved by the Research Ethical Committee of Kasr Al Aini in Dec 2015. All subjects who participated in the study gave their written consents. This study was conducted on 60 patients with ITP. Twenty-nine(48.3%) were males and 31 (51.7%) were females with a mean age 39.23 ± 12.49 and 40 healthy subjects; 20 (50%) males and 20 (50%) females with a mean age 36.08 ± 9.4 years as a control group.

All subjects were recruited from the Hematology

outpatient clinic- Cairo University.

Patients were subjected to history taking including the severity of the bleeding and need for platelet transfusion, history of drug therapy, and splenectomy. Detailed clinical examination, and laboratory investigation including CBC, liver function tests, bleeding, and coagulation profile. Exclusion criteria were other hematological diseases, Hepatitis C virus, HIV, recent viral infection, Helicobacter infection, autoimmune diseases as SLE, antiphospholipid syndrome, allergy, and liver disease.

Methods: Flow cytometry for platelet Annexin V and CXCR4 (CD184). FITC Annexin V apoptosis detection kit provided by BD pharmingen TM (catalog number 556547). Monoclonal CD184 (CXCR4 – PE) antibodies provided by Miltenyi Biotec: catalog number (130-098-354), clone 12G5 (isotype: mouse IgG2a). Staining with Annexin V is typically used in conjunction with propidium iodide (PI) for the identification of early and late apoptotic cells. Intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. Viable cells with intact membranes exclude PI. Therefore, cells considered viable are both Annexin V and PI negative, while cells that are in early apoptosis are Annexin V positive and PI negative, and cells that are in late apoptosis or already dead are both Annexin V and PI positive. All samples were analyzed on a colter EPICS profile II flow cytometry. Results were expressed as a percentage of early apoptotic cells, late apoptotic cells, living cells, and necrotic cells within the gated population. For CXCR4, results were expressed as a percentage of platelets expressing CXCR4 (CD184) within the gated population.

Statistical analysis

Data were analyzed using the SPSS (ver. 15). Data are presented as median values and ranges. Comparison between quantitative variables was done using the student *t*-test. All tests were two-tailed and considered significant at $p < 0.05$ and highly significant at $p < 0.001$. The relationship between the 2 variables was assessed using Pearson Correlation. A probability value of $P < 0.05$ was

considered statistically significant.

Results:

This study included 60 patients with immune thrombocytopenic purpura, forty-six (76.7%) of them were chronic and 14 (22.3%) were recently discovered to have the disease.

Steroids were the main therapy for 58 (96.7%) of the patients, only 10 (16.6%) patients had platelet transfusion and other immunosuppressive medications. Forty healthy subjects were enrolled in the study as a control group. Table 1 demonstrates a comparison between patients and control in demographic and laboratory parameters. Patients and control were age and sex-matched.

There was a highly significant difference between the two groups as regards the HB, HCT, and platelet count ($p < 0.001$).

Table (2) demonstrates a comparison of the Percentage of Annexin V and CXCR4 expression in the studied groups.

The percentage of the platelet with early apoptosis-positive markers (Annexin-D1) was less frequent in the ITP group compared to the control group ($p < 0.001$). However, living platelet (Annexin-D3), necrotic platelet (Annexin-D4) were more frequent in the ITP group compared to the control group ($p < 0.001$, $p = 0.016$) respectively. The percentage of the platelet with late apoptosis-positive markers (Annexin-D2) were not statistically different between cases and control ($p = 0.062$)

The percentage of the platelets with CXCR4 expression was lower in chronic ITP patients compared to the controls ($p < 0.001$).

No significant difference in the percentage of the platelet with positive Annexin or CXCR4 expression between males and females in both groups was found. Also, no significant difference was found in the percentage of the platelet with positive Annexin or CXCR4 expression between chronic and recently discovered patients ($p > 0.05$). There was a significant positive correlation between HB and platelet count in the ITP group ($r = 0.604$, $p < 0.001$).

Discussion

The most accepted theory for platelet destruction in ITP is antibody-mediated in the activated reticuloendothelial system (29-31). Previous studies on Annexin V and CXCR4 involved children with ITP. This study concerned adult ITP in whom the pathogenesis and fate are complex as we concluded in a previous publication where we found a great role of T cells in the pathogenesis of ITP development and impact of steroid treatment on the levels of cytokines release in these cases (32).

In this study, twenty-nine of our patients with ITP were males (48.3%) and thirty-one were females (51.7%) with age ranged from 19-61 (mean age 39.23 ± 12.49), this is in agreement with Silverman et al. who stated that the peak prevalence of ITP in adults ranges between 20-50 years with a female to male ratio 2.6:1 (33).

In this study, the percentage of the platelet with early apoptosis-positive markers (Annexin -D1) was less frequent in the ITP group compared to the control group with a highly significant difference ($p < 0.001$). In another study, thrombocytopenia induced by antiplatelet antibodies was associated with increased apoptotic markers (30). This difference could be due to that most of our patients were chronic, not acute cases.

However, in another study on children with ITP, no difference in the platelet percentage that became Annexin V positive between the patients with pediatric chronic ITP and the controls ($P = 0.05$). (34) They also found that the remaining studied platelets in circulation were apoptotic resistant with the previous destruction of the non-resistant platelets. They found that antibody-mediated platelet destruction via apoptotic pathway mainly occurs in the acute form of ITP. [35]

These findings are concomitant with our observations in adult ITP patients as most of them were chronic. And this was confirmed by the percentage of the platelet with late apoptosis-positive markers (Annexin-D2), living platelet (Annexin-D3), necrotic platelet (Annexin-D4) were more frequent in the ITP group compared to the control group ($p = 0.062$, $p < 0.001$, $p = 0.016$) respectively.

The platelets with CXCR4 expression were lower in chronic ITP patients compared to the controls with a highly significant difference ($p < 0.001$). This is contrary to Wang et al., who found increased CXCR4 expression on platelets in children with chronic ITP and they explained this by the SDF1-CXCR4 interaction triggering many intracellular signals related to platelet apoptosis resistance in chronic ITP patients (34). In this study, the decrease in platelet apoptosis might be responsible for the reduced CXCR4 platelet expression. Also, CXCR4 platelet expression is up-regulated by cytokines, including IL-2, IL-4, IL-6, and TGF- β , and down-regulated by INF- γ and SDF-1(34,35), these cytokines may be altered by the steroids which were the main line of treatment to our patient thus subsequently decreased the CXCR4 platelet expression.

Conclusion

Platelet Annexin V might be used as a marker of early apoptosis and together with CXCR4 expression may detect apoptosis resistance in adult patients with ITP. Due to the unpredictable course of ITP in adult patients and the possibility of its progress to refractory form, accurate choice of a biomarker is essential for evaluating prognosis, and detection of resistant forms is required.

Conflict of interest

The authors declare no conflict of interest.

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Table (1) Demographic and Laboratory data of patients group and control group.

	Patients (n=60)		Control (n=40)		p value
	Mean±SD	Range	Mean±SD	Range	
Age (years)	39.23± 12.49	19-61	36.08± 9.45	21-56	0.177
Male	29 (48.3%)		20 (50%)		0.870
Females	31(51.7%)		20 (50%)		
HB gm/dl	11.51 ±1.5	8.6-14.7	12.72±1.13	11-14.7	<0.001
Ht (%)	34.72±3.998	27.3-43.8	38.1±2.92	30.9-44.1	<0.001
RBCs (x10 ⁶ /L)	4.16±0.62	2.5-5.7	4.48±0.51	3.6-5.7	0.008
MCV (fl)	84.76±9.85	65.7-117	85.57±6.04	70-98	0.644
MHC (pg)	28.21±4.25	18.8-41.4	28.57±2.22	22.4-32.6	0.616
TLC (x10 ³ /L)	6.92±2.22	4-14	6.68±1.48	4-9	0.553
PLTs (x10 ³ /L)	50.5	9-127	266.5	190-330	<0.001

(HB = Hemoglobin, Ht= hematocrits, RBCs, red blood cells, MCV = means cell volume, MHC= means corpuscular hemoglobin,TLC = total leucocytes count, PLTs= platelets number, #=median)

Table (2) Percentage of Annexin V and CXCR4 expression in the studied groups.

	patients (no=60)		Control (no=40)		p value
	median	Range	median	Range	
Annexin-D1 %	0.085	0.00-2.48	1.20	0.00-5.76	<0.001
Annexin-D2 %	0.00	0.00-0.63	0.00	0.00-0.12	0.062
Annexin-D3 %	99.8	98-100	98.8	94-100	<0.001
Annexin-D4 %	0.07	0.00-2	0.00	0-0	0.016
CXCR4 %	0.42	0.07-5.59	1.4	0.07-36.8	<0.001

(Annexin -D1= early apoptotic cells, Annexin-D2= late apoptotic cells, Annexin-D3= living cells, Annexin-D4=necrotic cells, CXCR4= Chemokine Receptor type 4.)