Platelet-Associated CD154 in Immune Thrombocytopenic Purpura in Children

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Abstract: Background; CD40-ligand (CD154) is expressed on activated CD4+ T lymphocytes and is essential for the T cell–dependent activation of B lymphocytes. CD154 is also expressed at the activated platelet surface. Objective; to investigate the role of CD154 in ITP pediatric patients and correlate their levels with the course and progression of the disease. Subjects and Methods; This study included 25 patients with acute ITP (13 Females and 12 males) with age ranged between 2-6 years (group1).and 25 patients with chronic ITP (14 Females and 11 Males) with age ranged between 8 – 12 years (group II) also 25 apparently healthy children, (10 Females and 15 Males) with age ranged between 3-12years as control (group III). Studied groups were subjected to the following investigations; complete blood counts, bone marrow examination and flowcytometric analysis of CD154 B lymphocyte counts Results; We found that there was a highly significant increase in CD154in patients with acute ITP compared with chronic ITP and control group p0.001 and 0.0001 respectively) also there was a negative correlation between CD154 and platelet count in acute and chronic groups (r=-0.6 ,p=0.004 and r=-0.5, p=0.005 respectively) Also there was a positive correlation between CD154 and lymphocytic count in acute and chronic groups (r=0.422, p=0.007 and r=0.77 ,p=0.001 respectively), Conclusion; the increased number of CD154 might be one of the mechanisms that cause immune regulation dysfunction in ITP also the count is related to the severity of the disease as it was highly increased in acute phase than chronic and therefore CD154 expression is increased in ITP and is able to drive the activation of auto reactive B lymphocytes in this disease.
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Introduction

Immune (idiopathic) thrombocytopenic purpura (ITP) is an autoimmune disorder characterized by reduced platelet counts and normal or increased numbers of megakaryocytes in the bone marrow. Circulating antiplatelet autoantibodies are frequently detected. The most common target of antiplatelet antibodies is the platelet glycoprotein (GP) GPIIb/IIIa complex (integrin $\alpha IIb\beta 3$)(1) It is believed that T-cell activation is a critical event in ITP (1,2). T-cell help for B-cell activation relies on the interaction between CD154 and CD40. Interaction of CD154 on activated T cells, with its receptor CD40 on B cells, is essential for B-cell proliferation, differentiation, isotype switch, memory B-cell generation, and germinal center formation. (3,4). CD154 is expressed intracellularly in platelets. Normally, surface levels are low, but these increase after activation (5, 6)

Platelet CD154 has been studied mostly in its relation with the biology of the vascular endothelium and inflammation (7). Expression of CD154 by platelets could be critical in ITP because it would mean the coexistence at the platelet surface of potential target antigens, such as GPIIb/IIIa, and an essential costimulus of B-cell activation. This study aimed at evaluating the role of CD154 in ITP pediatric patients and correlates their levels with the course and progression of the disease.

Patients and Methods;

This is a cross sectional study that was conducted on 50 patients that were diagnosed as autoimmune thrombocytopenia (ITP) and were chosen from inpatients and outpatients in the hematology clinic of Suzan Mubarak Children Hospital, Al-Minia University, during the period from June 2009 to December 2010.

The patients included in the study were divided into 3 Groups. Group I include 25 patients with acute ITP (13 Females and 12 males) with age ranged between 2-6 years. Group II include 25 patients with chronic ITP (14 Females and 11 Males) with age ranged between 8 – 12 years. Group III control group including 25 apparently healthy children, (10 Females and 15 Males) with age ranged between 3-12years Inclusion criteria for patients with ITP:. Patients with thrombocytopenia (< 150.000/mm3) with normal white cell count, with no blast cell or abnormal cells in the peripheral blood. and without organomegaly or lymphadenopathy

All patients included in the study were receiving their proper treatment according to the standard protocol for management of ITP (mainly

corticosteroid for acute group and cyclophosphamide or the chronic group)

Both patients and controls were subjected to the following:

I- History taking: age, symptoms at time of presentation, preceding viral infection, drug intake, bleeding manifestations (type and site), and neurological symptoms.

II-Physical examination: particularly for presence or absence of bleeding manifestations, pallor, presence of lymphadenopathy and hepatosplenomegaly, presence of neurological signs.

III-Laboratory investigations:

1) Complete blood picture (CBC), using Automated Cell Counter.

2) Bone marrow examination for patients only.

3) CD 154(CD40L) counting in fresh blood by Flowcytometry.

Under complete aseptic conditions, 2 ml of venous blood were drawn from each subject on EDTA tube for complete blood count and flowcytometric assessment of platelet associated CD154. Washed cell are incubated with the fluorescein-labeled monoclonal antibody, which binds to B lymphocytes expressing CD40 Ligand. Unbound fluorescein-conjugated antibody is then washed from the cells. B cell expressing CD40 Ligand are fluorescently stained, with the intensity of staining directly proportional to the density of expression of CD40 Ligand. Cell surface expression of CD40 Ligand is determined by flowcytometric analysis using 488 nm wave length laser excitation.

Statistical analysis

After collection of data they were added and entered into a personal computer .Analysis of data was done using Standard computer program SPSS for windows, release 12.0 (SPSS Inc, USA). All numeric variables were expressed as mean ± standard deviation (SD). Comparison of different variables in various groups was done using student t test for normal and non parametric variables respectively. Person's and spearman's correlation tests were used for correlation normal and non parametric variables respectively. For all tests a probability (p) less than 0, 05 was considered significant. Graphic presentation of the results was also done.

The Results

Results of the present study were presented in the following tables and figures

Table (1): shows clinical manifestations in different groups, including: age, sex, purpuric eruption, epistaxis, and hypertension.

Table (2): It shows laboratory manifestations in different groups, including: platelet count/(ml) , WBCs /(ml) , hemoglobin (gm%),and lymphocytic count/(ml).

Table (3): shows comparison of CD154 % between different studied groups.

Figure (1) It reveals highly significant negative correlation between platelet count and CD154 in acute group (r = - 0.6, p = 0.004).

Figure (2): It reveals highly significant negative correlation between platelet count and CD154 in chronic group (r = -0.5 , p =0.005).

Figure (3): It reveals non significant and no correlation between platelet count and CD154 in control group (r = 0.04, p = 0.9).

Table (1): Clinical manifestations in different groups

Manifestation		Acute group (No=25)	Chronic group (No=25)	Control group (No=25)	P value
Age (y)	Range Mean±SD	2-6 3.7±1.32	**9-12** **10.64±1.11**	**2-12** 6.4±3	**0.001****
sex	Male Female	**12** **13**	**10** **15**	**15.** **10**	**0.36 NS**
purpuric eruption	Positive negative	**25 (100%)** **0 (0%)**	**25 (100%)** **0 (0%)**	**25 (100%)**	**0.001****
Pallor (mild to moderate	Positive negative	**8 (32%)** **17 (68%)**	**12(48 %)** **13 (52%)**	**25 (100%)**	**0.001****
epistaxis	Positive negative	**5 (20%)** **20(80%)**	**0 (0 %)** **25 (100%)**	**25 (100%)**	**0.005****
hypertension	Positive Negative	**1 (4%)** **24(86%)**	**9 (36%)** **16 (64 %)**	**25 (100%)**	**0.001****

N.B ** highly significant difference NS: non significant difference

There were highly significant differences in (age, purpuric eruptions, pallor, epistaxis, and hypertension) between the patients and controls. Hypertension present in one case was mild and mostly drug induced

Table (2): Laboratory manifestations in different groups:

	Range	Mean± SD	Range	Mean ±SD	Range	Mean ±SD	I vs II	I vs III	II vs III
Platelet count/ml	10000-37000	23320 ± 8610.6	66000-127000	92320 ± 21667.4	220000-420000	315600 ± 64683.8	0.0001**	0.0001**	0.0001**
WBCs count/ml	4200-13100	7800 ± 2471.5	4800-13600	8692 ± 11358.7	4000-11000	7292 ± 2074.3	0.65 NS	0.79 NS	0.47 NS
Hb (gm%)	7-12	9.7 ± 1.5	9.8-12	10.86 ± 0.69	11-16	12.7 ± 1.31	0.001**	0.0001**	0.0001**
Lymphocytic counts	1584-4704	2791.4 ± 929.2	1782-3864	2415 ±556.9	800-4400	2250.3 ± 992.6.6	0.12 NS	0.02*	0.49 NS

N.B. * significant difference ** highly significant difference NS: no significant difference

There was a highly significant decrease in the platelet count in the acute group compared to chronic and control groups, also in the chronic group there was a highly significant decrease in platelet count compared to control group As regarding the WBCs count there was no significant difference between any groups. There was a highly significant decrease in Hb level (gm %) in the acute group when compared to chronic and control groups, also in the chronic group there was a highly significant decrease in Hb (gm %) when compared to control group. There was a significant difference in lymphocytic count in the acute group compared to the control group.

Table (3): Comparison of CD154% between different groups

CD154%		**Group I (Acute)**	**Group II (Chronic)**	**Group III (Control)**
Range		29.07-522.2	30.2-191.1	4-66
Mean ±SD		152.69±128.64	76±39.13	23.48±14.98
P-value	**I vs II**	0.001**		
	I vs III	0.0001**		
	II vs III	0.02*		

There was a highly significant difference in CD154 in patients of acute group when compared to chronic and control groups, also there was a significant difference in CD154 in chronic group when compared to control group.

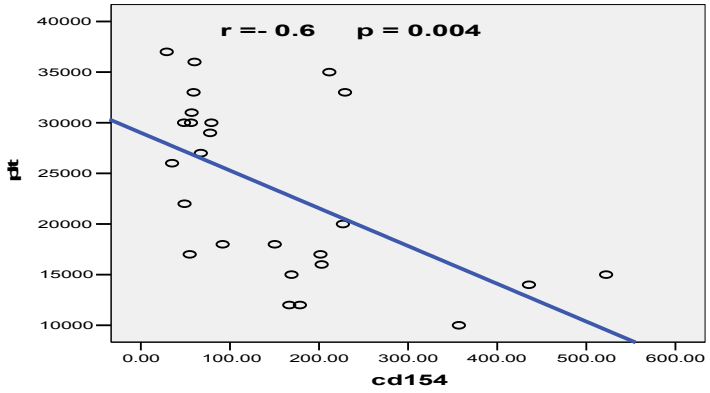

Figure (1) Correlation between platelet count and CD154 in acute group

It reveals highly significant negative correlation between platelet count and CD154 in acute group (r = - 0.6, p = 0.004)

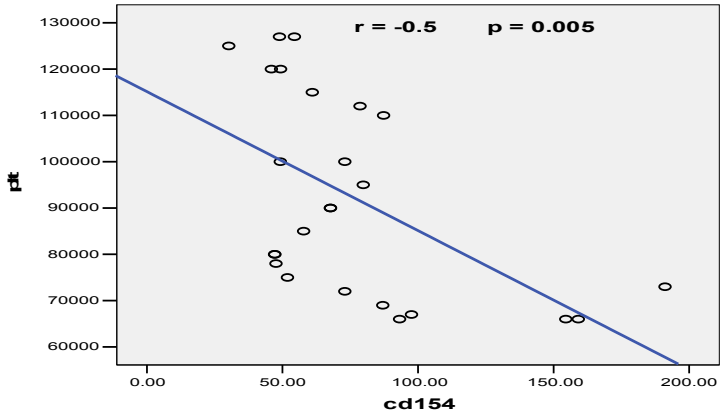

Figure (2): Correlation between platelet count and CD154 in chronic group

It reveals highly significant negative correlation between platelet count and CD154 in chronic group (r = -0.5 , p =0.005).

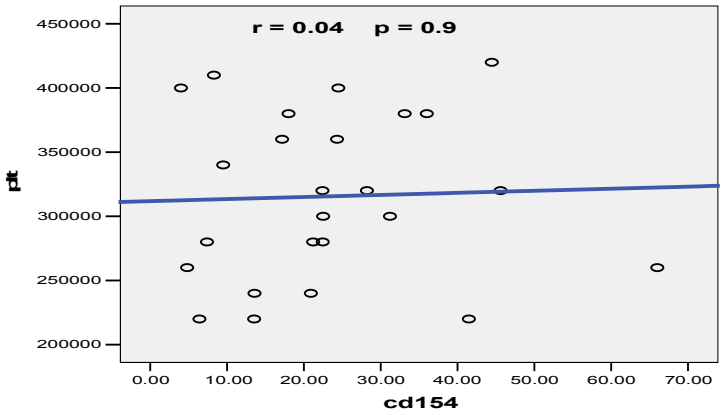

Figure (3): Correlation between platelet count and CD154 in control group

It reveals non significant and no correlation between platelet count and CD154 in control group (r=0.04, p=0.9).

Discussion

ITP is an autoimmune disease characterized by increased platelet destruction caused by anti-platelet autoantibodies, which mainly target a platelet surface antigen, GP IIb/IIIa, CD4 + T cells reactive with GP IIb/IIIa play a primary role in the disease process, since these autoreactive T cells are involved in the production of pathogenic anti-platelet autoantibodies. These findings are particularly useful for clarifying the pathogenic process in ITP patients and for developing a therapeutic approach that blocks pathogenic anti-platelet antibody production. (8)

CD40 is a cell surface receptor that belongs to the TNF-R (Tumor Necrosis Factor receptor) family, first identified and functionally characterized on B lymphocytes. CD40L (CD154,gp39), a transmembrane protein and a member of the TNF family, is expressed on activated CD4+T cells, mast cells, basophils, eosinophils, natural killer cells and activated platelets. CD40L is important for T cell-dependent B cell responses. The interaction of CD40L-CD40 is essential for T cell priming and the T cell-dependent humoral immune response. Therefore, interruption of the CD40-CD40L interaction with an anti-CD40L monoclonal antibody has been considered to be a possible therapeutic strategy in human autoimmune diseases. (9)

It is speculated that platelet-associated CD 154 is competent to induce the CD40-dependent proliferation of B lymphocytes. Therefore, platelet-associated CD154 expression is increased in ITP and is able to drive the activation of autoreactive B lymphocytes in this disease. Studies have shown that flowcytometry (FCM) is clinically useful for ITP patients, they denoted that their findings are particularly useful for clarifying the pathogenic process in ITP patients and for developing a therapeutic approach that blocks pathogenic anti-platelet antibody production. Blockade of the CD40/CD154 signal is a potential immuno-modulatory strategy for T cell-mediated diseases, and many findings suggest that CD40/CD154 blockade

therapy is potentially effective for ITP through selective suppression of autoreactive T and B cells to platelet antigens. (10)

In our work, we studied the surface expression of the activating molecules of CD154 on CD4+T lymphocytes of peripheral blood (PB) of patients with ITP, using Flowcytometry (FCM) for measuring them, to clarify their role in the mechanism and treatment of the disease. Our study showed that there was a significant correlation among the studied groups by comparison of their age, pupuric rash, pallor, epistaxis and hypertension. The results of the present study showed that chronic ITP occurs more commonly in old children than in young children with a more insidious onset of symptoms, higher frequency of female gender with a mean age for children, with acute ITP was 3.7±1.32 while for chronic ITP it was 10.46±1.11 (Table 1) and this was in agreement with Lanzkowsky et al., (11) who noticed that ITP in acute form is more common in children (2-6) years while chronic form is common in adults.

Another significant relation between age and ITP also noticed by Sutor et al., (12) and Kühne et al., (13) who noticed that the annual incidence of acute ITP is estimated to be 2.5 to 5 per 100.000 children and the peak age for presentation is 5.5 years. Gupta et al., (14) found that the peak age of manifestations is 2-5 years and both sexes are equally affected. But Hafiz, et al (15), Kühne et al., (13) and another study done by Robb et al., (16), observed that male children with ITP were predominant and this was against us. Bolton-Maggs (17) stated that adolescents particularly girls may pose a more complex challenge they are more likely to have chronic ITP, to have associated auto immune disorders and to have menorrhagia, Nugent (18) found that there is an equal incidence of ITP in both males and females in 1-7 years old age group in acute ITP of childhood ,these features are distinctly different from the adult form of the disease which is more likely to be chronic with a much greater incidence in females and no seasonal predilection.

Rapid diagnosis, reasonable care plan and education or rapid diagnosis, course of the disease and recent protocols used for management of ITP may be predisposing factors that can explain this phenomenon, however in young children particularly in infants, males are more often seen than female. In our study we found that a typical cases, the child is well and had a dramatic presentation with wide spread cutaneous purpura in all cases, acute and chronic and sometimes accompanied by epistaxis and pallor as a result of bleeding but they recover quickly without serious morbidity.

Although patients often present with bruises, petichiae and some mucosal bleeding, the incidence of life threatening hemorrhage (0.2-0.9%) but can be fatal when presenting with vital organs. The hemorrhagic manifestations depend on the degree of the thrombocytopenia and of platelet functions, The bleeding manifestations may also be affected by environmental and ethnic factors kühne et al., (19), however major hemorrhage as defined by intracranial or other overt internal or mucous membrane bleeding resulting in anemia (20).

Also Panepinto and Brousseau (21) noticed that approximately 75% of patients have mild symptoms at their initial presentation of ITP including the majority of children with platelet counts less than 10×109 /L. Mild ITP is defined as petichiae and bruising of the skin ,with or without some minor epistaxis and bleeding of short enough duration that there is no significant change in the child's daily activity, and in our study the high significant decrease in Hb level(gm%) in acute group when compared to chronic and control groups, also in chronic group the highly significance decrease in HB(gm%) when compared to control group are explained by presence of concomitant hypochromic microcytic anemia due to iron deficiency in acute and chronic groups and not due to bleeding tendency as bleeding was in the form of mild bruising, ecchymoses and pupuric eruption without sever or marked orificial bleeding.

In our study we found a highly significant relation between hypertension and ITP patients particularly chronic ITP (Table 1)and this was in agreement with Gupta et al., (14), who noticed that there are many side effects of corticosteroids that can occur related to the dose and duration ,one of them is hypertension in addition to others as weight gain, cushingoid facies and hypoglycemia. Nugent (18) has reported that hypertension is one of the common side effects of prednisone during treatment , also Panepinto and Brousseau (21) have reported that there are many complications of treatment with corticosteroids to acute ITP using the traditional dose which is (1-2) mg/kg of oral prednisone per day for 1 to 3 weeks. in addition to behavior changes, glucosuria, osteopenia, increasing appetite and weight gain.

In our study we found that there was a highly significant correlation between the platelet number and type of ITP ,where in acute ITP the platelet number was (10,000-37,000/mm3) while in chronic ITP the platelet number increase with the duration of ITP, as after 6 months , some increase in platelet count occurs in chronic ITP more than of acute ITP , and this was in agreement with Kühne et al., (22),

Imbach et al., (23) and Panepinto and Brousseau (21). Also Bolton-Maggs (17) reported that about 400-500 cases of acute ITP occur in UK each year and the child has usually platelet count of less than20×109/L. Blanchette et al., (24) showed from his experience that platelet count is below 150 ×109/L for more than 6 months used to define the chronic state and that was in agreement with our results in chronic ITP Also Nugent (18) has reported that platelet count with chronic ITP usually remains safe within range of more than (20,000/mm3) and this supports our results concerned platelet count in chronic ITP.

Our study has focused on the expression of CD154 during ITP and the results showed that there was increase in number of the expressed CD154 in ITP patients (Table 3), with a high significance between them (both acute and chronic) and the healthy group with a mean of (114.3±101.8) and (23.5±15) respectively. Also there was more expression of CD154 among patients with acute ITP when compared with chronic ITP with mean ± SD (152.69±128.64)and (76±39.13) respectively. These results really have been supported by many investigators:

Meabed et al., (25) ,have studied the expression of the activating molecules CD154 on CD4+T lymphocytes and CD40 on CD18+B lymphocytes of the peripheral blood of patients with ITP to clarify their role in the mechanism and treatment of this disease, his study included 30 patients with acute ITP and 30 patients with chronic ITP and 20 healthy subjects taken as controls, they have found that a highly statistically significant increases were demonstrated in percentages of CD154 and CD40 in all ITP patients (acute and chronic) when compared to controls (p value <0.001).the same finding was detected in acute ITP patients when compared to chronic ITP patients(p value <0.001). Solanilla et al., (10) , stated that CD154 /CD40L is expressed on activated CD4+T cells and is essential for T-cells dependant activation of B-lymphocytes, they showed that platelet associated CD154 is increased in ITP ,a disease characterized by an autoimmune response against proteins of platelet membrane, they found that platelet associated CD154 is competent to induce the CD40 dependent proliferation of B-lymphocytes and they observed an in vitro CD154 dependent production of antibodies to GPIIbIIIa complex when platelets and peripheral blood B-lymphocytes from ITP patients with circulating anti GPIIbIIIa antibodies were cultured together ,therefore platelet associated CD154 expression is increased in ITP and is able to drive the activation of autoreactive B-lymphocytes in this disease. Nagahama et al., (26), stated that they investigated levels of soluble CD40L (sCD40L) in ITP patients, in order to determine the influence of CD40-CD40L interaction on the pathogenesis of ITP, Thirty-eight of the 65 ITP patients (58.5%) had elevated levels of sCD40L. They found significant decreases in platelet counts in sCD40L +ve ITP patients.

Although the sCD40L level did not differ significantly between the control and non-immune thrombocytopenia groups, but among ITP patients. sCD40L level was significantly higher in those with untreated ITP than in those with treated ITP. Their findings suggested that there are two groups of ITP patients, one with elevated levels of sCD40L and one with normal levels of sCD40L. ITP cases in which sCD40L were increased appeared to involve changes in platelet counts. The pathogenesis of ITP may in some patients include alterations of the CD40/CD40 pathway. Kuwana et al., (27) have showed that platelet-reactive T-lymphocytes have been found in the blood of patients with this disorder with the major target antigens being platelet membrane glycoprotein IIb/IIIa autoreactive CD4+T lymphocytes and found in lesser number in blood of healthy controls.

In conclusion, our study suggests that increased number of CD154 might be one of the mechanisms that cause immune regulation dysfunction in ITP. Furthermore, our study confirm that the count of CD154 is considered to be related to the severity of ITP as it is significantly increased in active phase of the disease and decreased in the control. Therefore, count of CD154 might be a helpful diagnostic predictor of onset and improvement of ITP in children. Finally, we suggest that the interruption of the CD40-CD40L interaction with an anti-CD40L monoclonal antibody has been considered to be a possible therapeutic strategy in human autoimmune diseases as ITP.

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