Reduced L-Selectin Expression with Increased Anti-Apoptotic Protein in the Lymphocytes of Rheumatoid Arthritis Patients

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Abstract

Background: Rheumatoid arthritis is a chronic inflammatory disease of the synovium with uncertain pathogenesis. A number of phenotypic and functional T-cell defects have been described in rheumatoid arthritis.

Objective: To evaluate the cellular expression L-selectin (CD62-L) protein in the peripheral blood lymphocytes, L-selectin cellular expressions with the disease activity. And correlate it with the expression of Bcl-2 and P53 oncoproteins.

Patients and Methods: This study involved forty-six rheumatoid arthritis patients 42 female and 4 male, ages ranged from 30-60 years collected from AL-Kadhemia teaching hospital were examined and compared with 17 healthy control individuals of similar ages. Lymphocytes were separated from peripheral blood samples, the assessment of their cellular expression of cluster of differentiation 3, L-selectin, P53 and Bcl-2 by immunocytochemistry staining method.

Results: The results showed abundant accumulation of CD3 T lymphocytes in the peripheral circulation of rheumatoid arthritis patients in comparison with controls; that associated with decreased L-selectin expression in rheumatoid arthritis group. And a highly significant increase in the expression of Bcl-2 in the lymphocytes of rheumatoid arthritis patients as compared with the control group ($p<0.001$), however there was no significant difference in the expression of P53 between rheumatoid arthritis patients and controls ($P= 0.278$). The expression of L-selectin is negatively correlated with that of Bcl-2 ($r= 0.401$), while there was no significant correlation with P53 expression ($r=0.144$).

Conclusion: This study showed an increase in the peripheral blood T lymphocytes from patients with rheumatoid arthritis that could be resulted from loss of homing receptor, and increase in anti-apoptotic protein (Bcl-2).

Key words: Rheumatoid arthritis, L-selectin, lymphocytes, Bcl-2, P53.

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Received: 6th December 2015

Accepted: 29th December 2015

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Introduction

Rheumatoid arthritis (RA) is an autoimmune disease with characterized by dysregulated lymphocyte apoptosis process

[1, 2]. Apoptosis also has anti-autoimmune mechanism that deletes potentially pathogenic autoreactive lymphocytes, and limits tissue damage in autoimmune diseases including RA [2, 3].

It's strongly suggested driven by specific T-cell-mediated cellular immunity against self-antigens and the T-cell-mediated cellular immunity proposed to be involved in RA pathology [4]. Meanwhile, it has been recently proved that the increased number with abnormal differentiation pattern observed in peripheral lymphocytes in RA patients might be related to an abnormality in the apoptotic pathway [2,5 .[B-cell lymphoma -2 (Bcl-2) oncoprotein are known to play a central role in the regulation of apoptosis. Tumor suppressor (P53) protein is a tumor-suppressor gene that controls cellular proliferation. In its natural form (wild-type) P53 can bind to DNA and prevent cells from entering the S (synthetic) phase of the cell cycle so as to allow time for DNA repair. Alternatively, P53 dependent events can eliminate the cells by sending them down to an irreversible apoptotic pathway. Thus P53 allows the DNA either to be repaired or ultimately destroyed before replication renders the damage permanent [6].

On the other hand, the mitochondrial-mediated apoptosis is partially controlled by the family of Bcl-2 proteins, one of the biologically most relevant classes of apoptosis regulators [7]. The Bcl-2 protein was originally identified as the primary cause of some B-cell lymphoma (hence the designation Bcl), but was subsequently found to have strong anti-apoptotic activity in a variety of cell types including lymphocytes [8]. Upregulation of Bcl-2 proteins may cause systemic autoimmune disease through accumulation of activated T lymphocytes [8, 9].

L (leukocyte)-selectin (CD62-L) is a cell surface glycoprotein present on all types of circulating human lymphocytes CD62-L that is expressed on the surface of leukocytes supports lymphocyte rolling in peripheral lymph nodes [10,11]. The requirement for L-selectin for inflammatory responses has been addressed by a number of studies using gene knockout (KO) mice. Targeted mutation of L-selectin had a negative impact on acute inflammatory reactions [12].

The present study aims to evaluate the expression and correlation of CD62-L with P53 and Bcl-2 expression in PBLs isolated from RA patients regarding their increased number in peripheral circulation.

Patients and Methods

Patients and controls

The study groups consisted of forty-six Iraqi patients with RA fulfilled the American College of Rheumatology (ACR) classification criteria [13] were recruited from the out-patient clinic at the Department of Rheumatology and Rehabilitation, Al-Kadhumyia Teaching Hospital in Baghdad during the period from 4th of October 2005 to 14th of April 2006. Also, 17 apparently healthy controls were age and gender matched were enrolled in the study .

The scoring system of present disease activity was done according to modified DAS28-3 that combines of both clinical and laboratory parameters. The clinical examination of joint swelling and tenderness was performed for 28 joints (include the same joints: shoulders, elbows, wrists, metacarpophalangeal joints, proximal interphalangeal joints and the knees [14]. While the general immunolaboratory assessments included erythrocyte

sedimentation rate (ESR), C-reactive protein (CRP), and rheumatoid factor blood test (RF). Clinical and laboratory characteristics of the patients included in the study was recorded.

Blood samples and slides preparation

A blood sample (Five ml venous blood) was aspirated from a suitable vein from all patients and unaffected controls. Blood was collected in pyrogen-free silicone-coated tubes with heparin. The blood samples were used for lymphocyte separation according to Isopaque-ficoll technique (originally described by Boyum in 1968) [15].

Heparinised peripheral blood was diluted 1/1 with phosphate buffered saline (PBS), and mononuclear cells were isolated by ficoll density gradient centrifugation at 2000 rpm for 20 minutes. Mononuclear cells were washed three times with PBS for 5 minutes, resuspended at 1 x 106 cells/ml, and fixed on poly-L-lysine-coated glass slides, wrapped, and kept at -20°C until assayed .

The percentage of PBLs reactivity was semiquantified by immunocytochemistry staining method. Briefly, these precoated charged slides were removed from freezer, allowed to reach room temperature, unwrapped and then dipping the slides into PBS-filled jar for about 5 minutes and slides were placed on a flat level surface, then endogenous peroxidase was quenched by initial incubation of the smears by enough drops of Peroxidase block for 5 minutes at room temperature then rinse with PBS from a wash bottle, slides then placed in PBS wash bath for 2 minutes and excess buffer were taped and wiped around smears. Then, enough protein block reagent was applied for 5 minutes and excess blocking reagent were taped but not washed to ovoid non-specific binding of antibodies. Then, Coated lymphocytes were covered by 20 µl of 1/30 diluted mouse monoclonal Ab (primary Ab) specific for human CD3 (product no. AMAB90876, Sigma), L-selectin (product no. S13000, Sigma), P53 (product no. M700129, Dako) and BcI-2 (product no. M088729, Dako) proteins and incubated at 37°C for 1hour, then unreacted monoclonal Ab was removed by three cycle of washing with PBS each two minutes, then slides were washed wiped around the smear.

After that enough solution of biotinylated secondary antibody (anti-mouse Ab) were applied to cover each smear, distributed evenly over the precoated slides then placed in humid chamber for 1 hour at 37°C and washed in buffer and bathed in PBS for 5 minutes then wiped around smear. Enough solution of streptavidin conjugated peroxidase were applied to cover the smear and slides were placed in humid chamber for 1 hour at 37°C then washed in buffer and bathed in PBS for 5 minutes then wiped around the wells.

Then enough drops of freshly prepared DAB working solution were applied to cover the section at room temperature for 10 minutes or until the color is observed then the reaction terminated by rinsing gently with distilled water from a washing bottle. Slides then placed in bath of hematoxyline for 30 seconds at room temperature. Slides were rinsed gently with distilled water from a wash bottle then rinsed under gently running tap water for 5 minutes. A drop of mounting medium (DPX) was placed onto the wet smear and the spot quickly covered with a cover slip. Slides were let to dry and examined under 400X-magnification power of light microscope (ZEISS). The dark brown (homogenous or membranous) staining identified positive labeled cells only in results.

Statistical analysis

The percentage of each of the tested marker expression on PBLs was calculated by a simple calibration of percentage of reactivity as following formula: Percentage

of expression= (No. of positive cells/ total No. of cells) ×100% .

Statistical differences were analyzed using Independent sample-test. P-values <0.01 were considered statistically significant. Spearman correlation was used to assess the relationship between studied variables.

Results

The study included forty-six RA patients (four men and forty-two women), mean age (47.67 years) ranged in age from (25-66 year) with mean disease duration (6.5 years). Our patients were classified according to DAS into two main group the majority of them, 37 patients (80.4%) presented with high disease activity and the remainder were minimum disease group consist of 9 patients (19.6%).

Among RA patients 34 (73.9%) were seropositive and only 3 (21.4%) among control group. CRP serum concentration shown that RA patient's serum have higher level when compared with that of control groups. ESR concentration among RA showed highly significant difference when compared with control group and it was elevated in all RA patients.

Table (1): Characteristics of patients and control data are presented as means ± SD.

Parameters	Controls	RA patients	P-value
Women/men	15/2	42/4	>0.05
Age (mean±SD)	48.6±10	47.67±12.09	>0.05
RF sero-positive (No. (%))	3(21.4%)	34 (73.9%)	<0.001
ESR (mm/1st h) (mean±SD)	12.5±3.31)	67.43±20.26)	<0.001
CRP (mg/l) (mean±SD)	10.20±15.24	43.95±55.07	<0.001
Tender joints (mean±SD)	-	10.58±5.42	-
Swollen joints (mean±SD)	-	7.35±4.52	-
DAS-28(3) (mean±SD)	-	5.77±0.83	-
Disease duration (months) (mean±SD)	-	88.61±72.88	-
Duration of morning stiffness (minutes) (mean±SD)	-	76.41±41.30	-

The percentage of peripheral blood T cell population was indicated by CD3 reactivity. In our study we found significantly elevated percentage of CD3 positive cells in RA patients when compared with healthy controls ($p \leq 0.001$). While, highly statistical significant difference ($p \leq 0.001$) was found in L-selectin membranous expression (Figure 1), that indicated by lower expression in patients group than those of control group as shown in Figure (2). Also, there was highly significant overexpression of Bcl-2 in RA patient than those from control group ($p \leq 0.001$), and no significant difference in the P53 expression between RA patients and control groups (p=0.278) as shown in table 2.

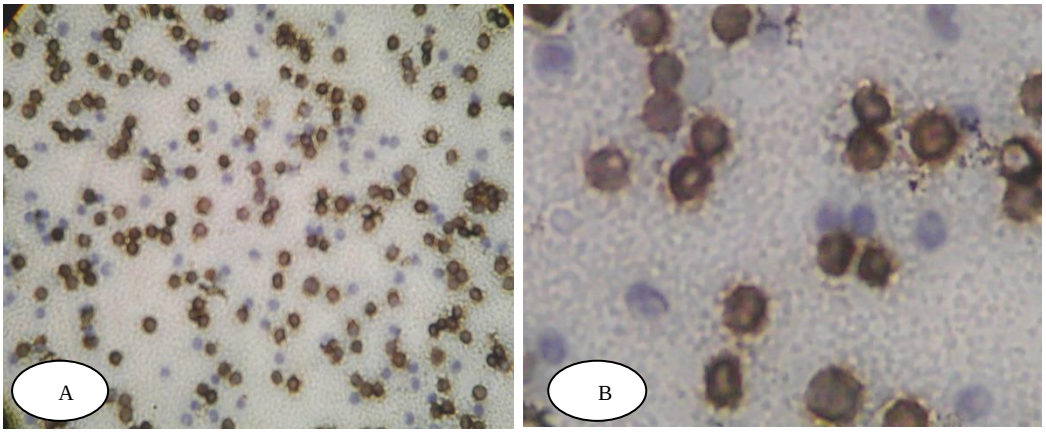

Figure 1. Immunocytochemical staining of PBL from RA patients stained with anti-human L-selectin mAb, visualized by peroxidase/DAB (brown) and counter stained with hematoxylin. A: Low power magnifications of 400X. B: High power magnifications of 1000X.

Table (2): Mean±standard deviation of PBL cellular expression of CD3, CD62-L and apoptosis regulating proteins (Bcl-2 and P53) in RA patients and control group.

Parameters	RA patients (mean±SD)	Controls (mean±SD)	P- value
CD3	79.22±1.42	72.92± 0.44	≤0.001
L-selectin	19.41±1.47	40.33±1.38	≤0.001
Bcl-2	34.17±0.97	20.5±1.026	≤0.001
P53	3.26±0.32	2.17±0.68	0.278

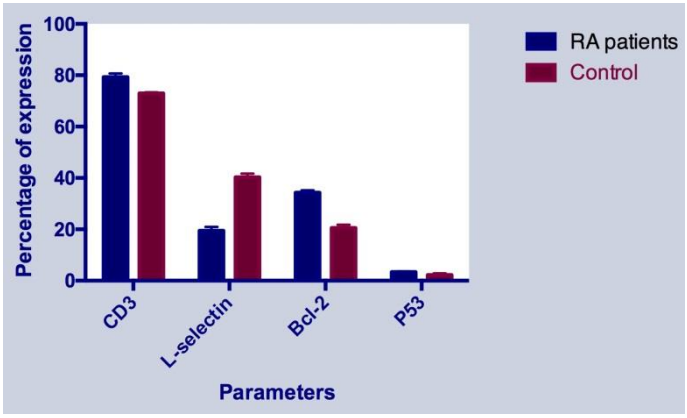

Figure (2): Bar chart showing mean percentage of expression of studied markers in study groups.

In RA patients and according to disease activity groups, we compared the percentage of expression of studied markers and found that no significant statistical differences in CD3, L-selectin, Bcl-2 and P53 expressions (p≥0.05) as shown in table 3, figure 3.

Table(3): Mean±standard deviation of PBL cellular expression of CD3 and apoptosis regulating proteins (Bcl-2 and P53) in high and minimum disease activity RA patient's groups.

Parameters	High disease activity (mean±SD)	Minimum disease activity (mean±SD)	P-value
CD3	79.26±1.33	79.04±1.8	0.686
L-selectin	39.20±1.587	43.91±2.61	0.147
Bcl-2	35.00±0.927	31.54±2.74	0.130
P53	3.20±0.525	3.45±1.14	0.823

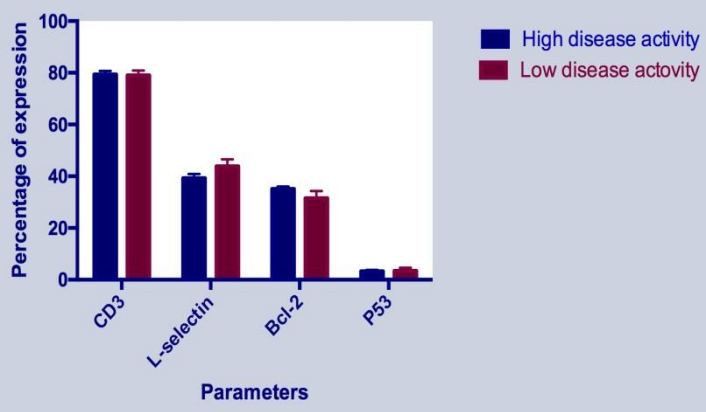

Figure (3): Bar chart showing mean percentage of expression of studied markers In RA patients.

Our study reported that there was an increased T-cell population in the peripheral circulation that associated with decreased expression of L-selectin. Significant negative correlation was found between CD62-L and Bcl-2. While, no correlation was found between L-selectin and P53, as shown in table 4.

Table (4): Correlation coefficient among studied markers

Parameters		Bcl-2	P53
L-selectin	*r*	-0.401	0.144

Discussion

Lymphocytes are key components of the immune cell infiltrate in the joints of RA patients, in which apoptosis may play divergent roles [16]. We previously reported that lymphocytes display abnormal activation state with elevated memory subset [17, 18].

Showing the importance of L-selectin as a major adhesion molecule on lymphocytes that regulate their homing to lymph nodes and entry into inflamed tissues in several experimental models of arthritis [19]. Our results showed the characteristics of atypical cells by marked decrease in L-selectin and higher number of CD3 T cells in peripheral circulation of RA patients in comparison with control groups. This result comes in agreement with an old study done by Bond and Hay in 1997 [20]. L-selectin down regulated from the T cell surface within

minutes after antigen receptor engagement and most antigen-activated T cells found in non-lymphoid organs are L-selectin low [21], which is in contrast with actively replicating cells that are immune-privileged sites like endometrium prepared for implantation showed very high expression of L-selectin, meaning that L-selectine is downregulated when there is immune activation or autoimmunity [22] .

Apoptosis is controlled genetically where P53 and Bcl-2 play a central role in its regulation [23-25]. Actively proliferating cells typically express Bcl-2 that protects them against apoptotic stimuli while terminally differentiated cells lose Bcl-2 expression, found that Bcl-2 protein in PBLs overexpressed and proposed a defect in the mechanism of deletion of over-produced lymphocytes that probably play role in the pathogenesis of RA [25-27] .

These data showed that the highly differentiated and apparently unstable state PBLs in RA may result in part from active inhibition of T cell apoptosis by environmental factors associated with the inflammation itself.

In conclusion, loss of CD62-L and defective lymphocyte apoptosis are playing an important role in RA inflammation. It could be due to their persistence producing the chronic characters of the disease.

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