Original Article

Immunocytochemical Detection of Glutathione S-Transferase in Peripheral Blood Lymphocytes of Rheumatoid Arthritis Patients

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Abstract:

Background: Peripheral blood lymphocytes (PBL)of Rheumatoid arthritis (RA)patients have a property of phenotypic and functional activation. Glutathione S- transferase pi (GSTπ) has been implicated in playing an important role in the initiation and progression of cellular activation.

Objectives: To determine the percentage of cellular expression of GSTπ in the lymphocytes of RA patients in comparison with controls and to explore the relation between its cellular expression and disease activity pattern.

Patients and Methods: This prospective study included46 RA patients and 17 healthy controls. Blood samples were taken and from all subjects PBL were isolated and then smeared on slides. The cellular reactivity for GSTπ was determined by immunocytochemistry technique.

Results: This study found lower expression of GSTπ in the RA patients with a statistical significant difference with control group, while no statistical difference was found in RA with high and minimum disease activity groups. No correlation was observed between GSTπ with Disease Activity Score (DAS).

Conclusion: Although the decrease in the expression of GSTπ in PBLs was pronounced in RA patients, however it doesn't correlate with disease activity state.

Keyword: Peripheral blood lymphocytes, Rheumatoid arthritis, Glutathione S-transferase, immunocytochemistry.

Introduction:

Oxidative modification of proteins and other biologic molecules leads to the expression of neoantigens, a possible first step in the development of autoimmunity, which may herald the future onset of clinically relevant autoimmune disease (1). Antioxidants, which mitigate tissue damage caused by reactive oxygen species, may serve important protective functions in RA. In addition to the effects of exogenous antioxidants, several enzymes, including glutathione S-transferase (GST), also regulate oxidation. A ubiquitous cytosolic protein, GST catalyzes the conjugation of glutathione to a variety of substrates, including reactive oxygen species (ROS) and other toxins, facilitating their elimination (1-3).GST, a 26-kDa protein, comprises a family of widely distributed heterogeneous cellular enzymes encoded by structurally different genes located on different chromosomes (4-6). In RApatients evidences exist for decreased levels of GSTπ in the synovial fluid T cells with a depletion of approximately 50% compared to osteoarthritic or healthy controls. It is thus evident how ROS, but overall, impaired GSTπ concentration, may contribute to cellular injury in rheumatoid arthritis and in other inflammatory/immune-mediated diseases. Indeed, a pathological status showing GSTπ depletion may not prevent the activation and
deposition of complement on the vascular endothelium, thus leading into inflammation, vascular injury and tissue damage (6-9). In lymphocytes, the reduced form of glutathione (GSH) has been implicated in playing an important role in the initiation and progression of cellular activation (6,10,11). Depletion of intracellular GSH inhibits lymphocyte activation by mitogens (12,13).

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Patients and controls:

The study groups consisted of 46 Iraqi patients with RA fulfilled the American College of Rheumatology (ACR) classification criteria (14,15), recruited from the outpatient clinic at the Department of Rheumatology and Rehabilitation, Al-Kadhumyia Teaching Hospital in Baghdad. In addition, 17 age-and sex-matched healthy controls enrolled in the study. These controls were healthy blood donors.

The scoring system of present disease activity was done according to modified DAS28-3, that combines both clinical and laboratory parameters. The clinical examination of joint swelling and tenderness performed for 28 joints (include the

same joints: shoulders, elbows, wrists, metacarpophalangeal joints, proximal interphalangeal joints and the knees (15,16). While the general immunolaboratory assessments included erythrocyte sedimentation rate, C-reactive protein, and RF.

Blood samples and slides preparation:

A blood sample (Five ml venous blood) aspirated from a suitable vein from all patients and controls. Blood collected in pyrogen-free silicone-coated tubes with heparin. The blood samples used for lymphocyte separation according to Isopaque-ficoll technique (originally described by Boyum in 1968) (17). 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, resuspendedat 1 x 10^6 cells/ml, and fixed on poly-L-lysine-coated glass slides, wrapped, and kept at -20°C until assayed.

Detection of lymphocytesGSTπ protein:

The percentage of PBLs reactivity was semi quantified 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 was taped and wiped around smears. Then, enough power block reagent (1/10 diluted in PBS) were applied for 5 minutes and excess blocking reagent was taped but not washed to ovoid non-specific binding of antibodies.Then, the coated lymphocytes were covered by 20 µl of 1/50 diluted mouse monoclonal Ab (primary Ab) specific for human GSTπ Slides then incubated at 37°C for 1hr, then, unreacted monoclonal Ab removed by three cycle of washing with PBS each for two minutes, then slides 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 was 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 substrate 3,3-Diaminobenzidine (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 hematoxylin for 30 seconds at room temperature. Slides 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 let to dry. Slides were examined by 400X-magnification power of light microscope (ZEISS). The dark brown (homogenous or membranous) staining identified positive labeled as shown in figure (2).

Statistical analysis:

The percentage of each of the tested marker expression on PBLs 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 <0.05 were considered statistically significant. The software used for statistical analysis was SPSS version 17.

Results:

The study included 46 RA patients (four men and 42 women), mean age (47.67 ±12.09) years ranged in age from (25-66) year with mean disease duration (88.61± 72.88) months. 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%)(Table 1).

Table 1: patients and control characteristics.data are presented as means ± SD.

	Controls	RA patients	P value	RA patients		P value
				HAD	MDA	
Women/men	15/2	42/4	>0.05	34/3	8/1	>0.05
Age	48.6±10	47.67±12.09	>0.05	48.06±11.96	46.45±12.97	>0.05
Disease duration (months)	----	88.61±72.88		92.34±68.28	76.73±92.67	>0.05
ESR (mm/1st h	12.50±3.31	67.43±20.26	<0.001	70.94±19.54	53±17.33	>0.05
CRP (mg/l)	10.20±15.24	43.95±55.07	<0.001	49.78±59.53	20±17.75	>0.05
Tender joints	-----	10.58±5.42	-	12.54±4.62	4.77±3.19	<0.001
Swollen joints	-----	7.35±4.52	-	8.63±4.36	3.66±1.80	<0.001
DAS-28(3)	-----	5.77±0.83	-	6.11±0.63	4.844±0.24	<0.001
RFsero-positive (No. (%))	3 (21.4%)	34 (73.9%)	<0.001	72.9%))27	6 (63.54%)	>0.05
Duration of morning stiffness (minutes)	-----	76.41±41.30	-	84±41.72	52.27±30.28	<0.05

ESR= Erythrocytes sedimentation rate, CRP= C reactive protein, DAS= Disease activity score, HDA= High disease activity group, MDA= Minimum disease activity group, RF= Rheumatoid factor.

Immunocytochemical expression of GSTπ protein in PBLs: GSTπ was detected as a proteinous form in PBLs of studies subjects (Figure 1).

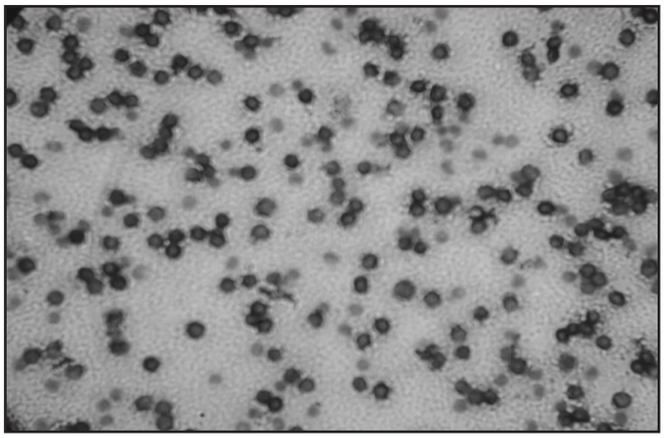

Figure 1: Immunocytochemical staining of PBL from Rheumatoid Arthritis patients stained with GST mAb, visualized by peroxidase/DAB (brown) and counter stained with Meyer's hematoxylin. Low power magnifications of 400X.

According to in-dependent sample t-test, there was a highly statistical significant difference ($p<0.001$) in mean of GSTπ protein expression in PBLs between RA patients and control group. In RA patients GSTπ shows lower percentage of expression than those of controls (Figure 2), (Table 2).

Table 2: Comparison of PBL cellular expression of GSTπ in RA patients and control group.

	Controls	RA patients	P value	RA patients		P value
				HDA	MDA	
GSTπ	44.49± 4.95	25.46± 10.98	<0.001	23.48±10.58	31.63±12.56	>0.05

HDA= High disease activity group, MDA= Minimum disease activity

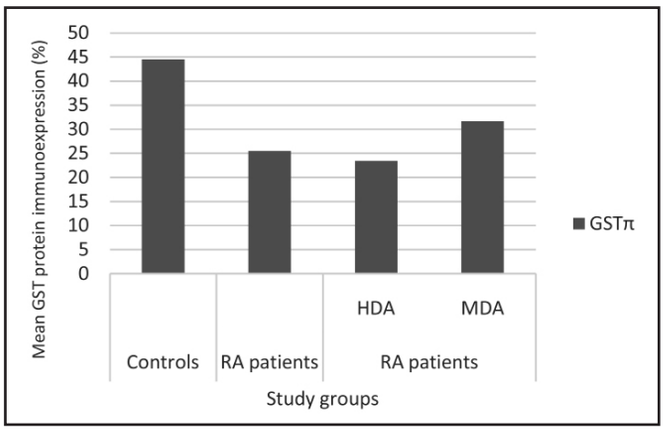

Figure 2: Cellular expression of GSTπ in peripheral blood lymphocytes measured by immunoytochemistry method. Bars represent mean of expression.

However, between high and minimum disease activity there is no statistical significant difference in mean percentage of expression of GSTπ ($p>0.05$), (Table 2). No correlation was observed between GSTπ with Disease Activity Score (DAS-28 (3)),(Table 3).

Table 3 Pearson Correlation between DAS-28(3) and GSTπ expression.

		DAS-28(3)
GSTπ	Pearson Correlation	-0.143
	Sig. (2-tailed)	0.342

Discussion:

The auto-immune diseases of rheumatoid arthritis (RA) demonstrate depressed T-cell function together with B-cell hyperactivity. The process of inflammation is associated with response to oxygen intermediates and free radicals released from macrophages and neutrophils (3,5,18,19).GSH is the cellular component responsible for the protection of cells from free radical damage (8,19,20). Thus, a chronic inflammatory process such as in RA would involve the utilization of GSH and potentially lower intracellular glutathione concentrations. The results of this study found that there is lower expression of GSTπ enzyme when compared to control group, this results supported by another studies done on RA patients in addition, RA isa chronic inflammatory condition which has been associated with low serum and erythrocyte GSH concentrations when compared to normal (3,5,19,21,22). Also, the null alleles of GST were found to be associated with increased susceptibility for developing RA especially in the east Asian populations (23,24).Studies in RA patients have demonstrated low serum thiol levels when compared to normal individuals. Thus, in both ageing and the auto-immune diseases of RA and SLE, low intracellular GSH pools may be playing a role in defective T-cell function(3,5,25). Impaired GSH concentration, may contribute to cellular injuryin RA and in other inflammatory/immuno-mediated diseases. Indeed, a pathological status showing GSH depletion may not prevent the activation and deposition of complement on the vascular endothelium, thus leading into inflammation, vascular injury and tissue damage (6-9).However, GSTπ lowered expression was pronounced as a main feature of lymphocyte defectiveness in RA patients and its disease activity was not related to GSTπ expression. In conclusion the decrease in the expression of GSTπ in PBLs was pronounced in RA patients, however it doesn't correlate with disease activity state.

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Haider F Ghazi: study conception & design

Asmaa B Al-Obaidi: working &drafting of manuscript

Huda Adnan: acquisition of data analysis

Manal A Habib: critical revision & final paper finishing & editing

References:

1. *Pattison DJ, Winyard PG: Dietary antioxidants in inflammatory arthritis: do they have any role in etiology or therapy? Nat ClinPractRheumatol 2008, 4:590-596.*

2. *Bae SC, Jung WJ, Lee EJ, et al: Effects of antioxidant supplements intervention on the level of plasma inflammatory molecules and disease severity of rheumatoid arthritis patients. J Am CollNutr 2009, 28:56-62.*

3. *Cerhan JR, Saag KG, Merlino LA, et al: Antioxidant micronutrients and risk of rheumatoid arthritis in a cohort of older women. Am J Epidemiol 2003, 157:345-354.*

4. *Islam MQ, Platz A, Szpirer J,et al. Chromosomal localization of human glutathione trans-ferase genes of classes alpha, mu, and pi. Hum Genet. 1989;82:338-42.*

5. *Mannervik B and Danielson UH. Gluthionetransferases. Stucture and catalytic activity. CRC Crit. Rev. BiochemMol Biol. 1988;23,:283-337.*

6. *Li YR, and Kauffman JM. Molecular Medicine of Rheumatoid Arthritis: From Molecular Pathphysiology to Novel Therapeutics and Evidence-Based Practice. Ann OrthopRheumatol. 2014;2(2): 1014-24*

7. *Collard CD, Agah A, Stahl GL. Complement activation following reoxygenation of hypoxic human endothelial cells: role of intracellular reactive oxygen species, NF-kB and new protein synthesis. Immunopharmacology. 1998;39:39–50.*

8. *McInnes IB, Schett G. The pathogenesis of rheumatoid arthritis. N Engl J Med. 2011; 365: 2205-2219.3.*

9. *Brusca SB, Abramson SB, Scher JU. Microbiome and mucosal inflammation as extra-articular triggers for rheumatoid arthritis and autoimmunity. Curr Opin Rheumatol. 2014; 26: 101-107.9.*

10. *Chaplin DD, Wedner HJ. Inhibition of lectin-induced lymphocyte activation by diamide and other sulfhydril reagents. Cell Immunol. 1978;36:303.*

11. *Keenan BT, Chibnik LB, Jing Cui J, et al. Effect of Interactions of Glutathione S-Transferase T1, M1, and P1 and HMOX1 Gene Promoter Polymorphisms With Heavy Smoking on the Risk of Rheumatoid Arthritis. Arthr and Rheum. 2010;62:3196–3210.*

12. *Fishman CM, Udey MC, Kurtz M, Wedner HJ. Inhibition of lectin induced lymphocyte activation by 2-cyclohexane- 1-one; decreased intracellular glutathione inhibits an early event in the activation sequence. J. Immunol. 1981;127:2257.*

13. *Hamilos D, Wedner HJ. The role of glutathione in lymphocyte activation. I. Comparison of the inhibitory effects of buthioninesulfoximine and 2-cyclohexane- 1-one by nuclear size transformation. J Immunol. 1985;135:2740.*

14. *Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum. 1988;31:315-24.*

15. *Aletaha D, Neogi T, Silman AJ et al. 2010 Rheumatoid Arthritis Classification Criteria. An American College of Rheumatology/European League Against Rheumatism Collaborative Initiative. 2010; 62:2569–81.*

16. *Fransen PL, van Riel CM. The Disease Activity Score and the EULAR response criteria. Clin Exp Rheumatol.2005;23:93-99.*

17. *Boyum, A. lymphocyte separation, Scan J Clin Lab Invest. 1968;21(97).*

18. *Fantone JC, Ward PA. Role of oxygen derived free radicals and metabolites in leukocyte-dependent inflammatory reactions. Am JPathol.1982;107:397.*

19. *Griffiths HR. Is the generation of neo-antigenic determinants by free radicals central to the development of autoimmune rheumatoid disease? Autoimmun Rev 2008;7:544–9.*

20. *Meister A. Selective modification of glutathione metabolism. Science.1983;220:472.*

21. *Fidelas RK, Tsan MF. Enhancement of intracellular glutathione promotes lymphocyte activation by mitogen. Cell Immunol. 1986;97:155.*

22. *Gambhir JK, Lali P, Jain AK. Correlation between blood antioxidant levels and lipid peroxidation in rheumatoid arthritis. Clin Biochem 1997;30:351–5.*

23. *Mikuls TR, Gould KA, Bynoté KK, et al. Anticitrullinated protein antibody (ACPA) in rheumatoid arthritis: influence of an interaction between HLA-DRB1 shared epitope and a deletion polymorphism in glutathione s-transferase in a cross sectional study. Arthritis Res Ther. 2010; 12: 213.*

24. *Ji JD, Lee WJ. Association between the polymorphisms of glutathione S-transferase genes and rheumatoid arthritis: a meta-analysis. Gene. 2013; 521: 155-159.*

25. *Lorber A, Pearson CM, Merdith WJ, Gantz-Mandel LE. Serum sulfhydryl determinations and significance in connective tissue diseases. Ann Intern Med.1964;61:423.*