Changes in Protein Sulfhydryls, Protein Carbonyls and Lipid Peroxidation Levels in Sera of Patients with Rheumatoid Arthritis: Correlation with Disease Activity

Romatoid Artrit'li Hastaların, Serum Protein Sülfidril, Protein Karbonil ve Lipid Peroksidasyon Düzeylerindeki Değişim: Hastalık Aktivasyonu ile İlişkisi

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Abstract

Objective: Reactive Oxygen Species (ROS) play a major role in the generation of acute and chronic inflammatory processes one of which is rheumatoid arthritis (RA).

Patients and Methods: This study was designed to investigate ROS and antioxidant affects in RA patients. The antioxidant activity was determined by measuring total protein sulfhydryl (SH) levels. ROS was indicated by measuring protein carbonyls and malondialdehyde (MDA) levels. A total of 29 RA patients aged between 31 to 68 years old were recruited. Also 20 control subjects were selected from healthy individuals Total protein SH, protein carbonyls and MDA levels were measured from sera of both groups.

Results: Total protein SH levels of RA patients were found to be significantly decreased than those found in control group (396,26±42,33 mmol/L vs 677,21±59,98 mmol/L, p<0,001). Also the levels of protein carbonyls and MDA were found to be statistically increased in RA patients respectively (105,45±14,26 mmol/L vs 93,65±10,49 mmol/L, p<0,05, 14,51±2,28 mmol/L vs 5,56±1,25 mmol/L, p<0,001). Patients active in disease had higher levels of MDA and lower levels of total protein SH levels when compared to those patients in remission. A negative correlation was found between protein carbonyls and erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) (r=-0,415, p<0,05, r=-0,687, p<0,001) in RA patients. No correlation was found in patients active in disease but negative corelation was found between protein carbonyls and ESR and CRP in patients in remission.

Conclusion: In conclusion the level of ROS seems to increase and measurement of these markers may give us information about inflammatory process in RA. *(Rheumatism 2006; 21: 18-22)*

Key Words: Rheumatoid arthritis, sulfhydryls, protein carbonyls, malondialdehyde

Özet

Amaç: Reaktif Oksijen Ürünleri (ROS), romatoid artrit (RA) gibi akut ve kronik inflamatuvar olaylarda önemli rol oynamaktadırlar. Bu çalışma RA'li hastalardaki ROS ve antioksidan etkinin varlığını araştırmak amacıyla planlandı.

Hastalar ve Yöntem: Antioksidan etkinlik total protein sülfidril (SH) düzeyi ile, ROS ise protein karbonil ve malondialdehid (MDA) düzeyleri ile belirlendi. Çalışmaya yaşları 31-68 arasında toplam 29 RA'li hasta alındı. Kontrol grubu aynı cins ve yaşta 20 sağlıklı bireyden oluşturuldu. Hasta ve kontrol grubunun serumlarından total protein SH, protein karbonil ve MDA düzeyleri ölçüldü.

Bulgular: RA'li hastalarda total protein SH düzeylerinde kontrol grubuna göre belirgin azalma olduğu gözlendi (396.26±42.33 mmol/L vs 677.21±59.98 mmol/L, p<0.001). Ayrıca sırasıyla protein karbonil ve MDA düzeylerinde de kontrol grubuna göre istatistiksel anlamlı artış olduğu tespit edildi (105.45±14.26 mmol/L vs 93.65±10.49 mmol/L, p<0.05, 14.51±2.28 mmol/L vs 5.56±1.25 mmol/L, p<0.001). Hastalık aktivasyonu olanlarda remisyondakilere göre daha yüksek MDA düzeyleri ve daha düşük protein SH değerleri olduğu görüldü. RA'li hastalarda protein karbonil düzeyleri ve eritrosit sedimentasyon hızı (ESR) ve C-reaktif Protein (CRP) düzeyleri arasında negatif bir korelasyon (r=-0.415, p<0.05, r=-0.687, p<0.001) saptandı. Aktif hastalığı olanlarda herhangi bir ilişki bulunmamasına rağmen remisyondaki hastalarda protein karbonil ve ESR ve CRP arasında negatif bir korelasyon tespit edildi.

Sonuç: Sonuç olarak RA'da ROS 'de artış görülmektedir ve bu biyokimyasal belirteçlerin ölçümü ile inflamasyon düzeyi hakkında bilgi edinilebilinir. *(Romatizma 2006; 21: 18-22)*

Anahtar Kelimeler: Romatoid artrit, protein sülfidril, protein karbonil, malondialdehid

Introduction

Rheumatoid arthritis (RA) is a disabling autoimmune disease characterized by chronic inflammation of the joints. For the majority of the cases the synovitis will lead to permanent damage of the articular cartilage and bone (1).

Reactive oxygen species (ROS) play a major role in the generation of acute and chronic inflammatory diseases (2). In patients with inflammatory joint diseases, many evidences implicating the role of ROS in the pathogenesis of acute and chronic inflammatory synovitis exist, especially in RA (3). Oxidative stress in the joints of RA patients usually increases the metabolic rate of synovial tissue and locally activates the leucocytes, which in turn lead to degradation of hyaluronic acid in the joints (4).

Detection of low antioxidant levels in sera of RA patients supports the contribution of free radicals in inflammatory processes. This study was designed to investigate the anti-

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oxydant activity and the role of ROS in RA patients. Also to examine the changes in total protein sulfhydryl (SH) levels, protein carbonyls as an indicator of protein oxidation and malondialdehyde (MDA) levels as an indicator of lipid peroxidation in sera of patients with RA. Correlation of these markers with disease severity was also evaluated.

Patients and Methods

Study population

Totally 29 RA patients (F/M=22/7) admitted to the outpatient clinics of the department of Physical Medicine and Rehabilitation were recruited for this study. Diagnosis of the patients with RA was based on the American Rheumatism Association (ARA) 1987 revised criteria (5). The clinical examination included the duration of morning stiffness, the number of swollen joints and the number of tender joints. Swollen and tender joint count included 28 joints and these were metacarpophalangeal, proximal interphalangeal joints, wrists, elbows, shoulders and knees (6). Routine biochemical analysis such as full blood count, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF) were performed. Also patients were asked for their medication schedule. They were allowed to continue to take their drugs such as corticosteroids, non-steroidal anti-inflammatory drugs (NSAID) and disease modifying antirheumatic drugs (DMARDs). Twenty control subjects (F/M=10/10) were selected from age and sex matched healthy individuals. The control subjects were free of any chronic diseases and were not on any medical therapy.

Biochemical analysis

The blood samples were collected after an overnight fasting. Total protein SH levels were measured spectrophotometrically, using Ellman's reagent, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), with the thiol-disulfide interchange reaction between DTNB and thiol providing the basis of the spectrophotometric assay (7). Protein oxidation status was assessed with a colorimetric assay that measures protein carbonyl content, after reacting the serum with dinitrophenylhydrazine, as described by Levine et al. (8). The end product of lipid peroxidation, MDA was measured to assess the degree of plasma lipid peroxidation, using the thiobarbituric acid (TBA) method (9).

Statistical analysis

Data were expressed as mean±standard deviation (SD) of mean. A nonparametric Mann Whitney U test was used to evaluate the differences between groups. Correlation analyses and multistep regression analyses were also performed by using SPSS for windows (version 10.0.1). A P value <0.05 was considered to be a minimum significance level.

Results

Table 1 depicts the baseline characteristics of the patients. The mean age of RA patients was 53.4 (31-68) years and the control group was 47.3 (35-62) years. The mean duration of disease was 7.04 (1-30) years. Twenty-four of the patients were seropositive. Duration of morning stiffness was 44±12.3 minutes. Number of tender and swollen joints were 8.7±1.3 and 2.4±0.5 respectively. Four patients were receiving chloroquine (400mgr/day), 6 patients were treated by methotrexate (15mgr/ week), 17 patients were treated by sulphasalazine (2gr/day) and 2 of them were receiving both methotrexate and sulphasalazine. In addition, 11 patients were receiving small doses of prednisone (5-10mg/day). According to clinical and laboratory parameters (ESR,CRP), 14 patients were active in disease. Twenty patients were diagosed as early RA (disease duration less than 2 years).

The mean value of RF Ig M (U/ml) was 62.8 (5-231), ESR (mm/hour) was 40(13-88) and CRP(mg/L) was 21.5 (2-70) for RA patients. All parameters were in normal range in control group.

Plasma total SH levels in RA patients were found to be significantly decreased than those found in control subjects (396.26±42.33 μmol/L vs 677.21±59.98 μmol/L, p<0.001). On the other hand, plasma protein carbonyls were found to be increased in RA patients (105.45±14.26 μmol/L vs 93.65±10.49 μmol/L, p<0.05). Almost five-fold increase in MDA levels were observed in RA patients when compared to control subjects (14.51±2.28 μmol/L vs 5.56±1.25 μmol/L, p<0.001) (Table 2).

According to clinical and laboratory measures, patients were either active in disease (n=14) or in remission (n=15).

Table 1. Baseline characteristics of patients. Values represent mean (range) or mean±standard deviation (SD) of mean or percentage where appropriate

	Patients (n=29)
Gender (F/M)	22/7
Age (year)	53.4 (31-68)
Seropositivity	82,8%
Duration of morning stiffness (min)	44 ± 12,3
Number of tender joints (0-28)	8.7 ± 1,3
Number of swollen joints (0-28)	2.4 ± 0,5
RF Ig M (U/mL)	62.8 (5-231)
ESR (mm/1st hour)	40 (13-88)
CRP (mg/L)	21.5 (2-70)

Table 2. Serum levels of total protein sulfhydryl (SH), protein carbonyls and malondialdehyde (MDA) in rheumatoid arthritis (RA) patients. Values represent mean±standard deviation (SD) of mean

	RA patients (n=29)	Controls (n=20)
Serum total SH (μmol/L)	396.26 ± 42.33*	677.21 ± 59.98
Serum protein carbonyls (μmol/L)	105.45 ± 14.26**	93.65 ± 10.49
Serum MDA (μmol/L)	14.51 ± 3.28*	5,56 ± 1.25

*p<0,001, **p<0,05

Statistical analysis revealed no significance in protein carbonyl levels between patients active in disease and patients in remission (103.57±10.33 μmol/L vs 105.36±1.41 μmol/L). However RA patients active in disease had lower levels of total protein SH (381.36±33,15 μmol/L vs 412.19±46.47 μmol/L, $p<0.05$) and higher levels of MDA (15.60±2.27 μmol/L vs 10.54±1.27 μmol/L, $p<0.001$) when compared to patients in remission. But all three parameters were also significantly higher in patients in remission than those found in control subjects.

Correlation studies were also revealed that no statistically significant differences were observed between different oxidative stress markers and biochemical markers, such as ESR, CRP or RF in patients active in disease but negative correlations were found in patients in remission between carbonyl levels and ESR and CRP levels ($r=-0.59$, $p<0.05$ and $r=-0.86$, $p<0.001$) respectively. Multiple regression analyses and equations obtained for total protein SH, protein carbonyls and MDA are shown in Table 3. There was only one significant independent factor which was CRP levels for protein carbonyls (Table 3)

Discussion

Oxidative stress is implicated in the pathogenesis of several disease states including aging, aging-related chronic diseases such as atherosclerosis, diabetes mellitus, ischemia-reperfusion injury and RA (1,2). Although it is not always possible to measure directly in biological systems, several biomarkers providing a measure of oxidative damage to biomolecules have been identified (10-12).

Rheumatoid arthritis characterized by focal loss of cartilage due to upregulation of catabolic pathways, induced mainly by pro-inflammatory cytokines and ROS (13). Oxidative stress contributes to joint inflammation and damage in RA. In normal conditions, the synovial cavity has a negative pressure. During exercises, vascular patency is maintained to allow the nutrition of the avascular cartilage. In a mobile inflammed joint, the cavity pressure is raised and upon movement this pressure exceeds the capillary perfusion pressure, causing collapse of the blood vessels. This exercise induced multiple episodes corresponds to hypoxia-reperfusion injury which consequently leads to the generation of redox environment (10). Several factors contribute to the production of free radicals on of which is NADPH mechanism (14,15). The production of these ROS oxidise IgG and induce rheumatoid factor production, oxidise hyaluronan and lead to the production of hyaluronan fragmentation products. Also oxidation of lipids generate aldehydes that are toxic to immune system (15). Besides these mechanisms, selenium deficiency has been shown to be related with several pathologies including immune function (16). Chondrocyte cell death has been shown to be a causative factor in the pathogenesis of RA. It has been reported that production of nitric oxide (NO) itself is not cytotoxic, even protective but cell death from NO occurs under conditions where ROS are also generated (17,18).

Previously, the changes in total protein SH levels were evaluated in RA patients. Decrease in serum non-protein SH levels were reported (19-21) and were found to be closely interrelated with disease duration and the age of the patients (20). On the otherhand, selenium dependent antioxidative enzymes were also evaluated and serum glutathione peroxidase (GSH-Px) and glutathione reductase (GR) activities were found to be decreased (11,22) and glutathione-S-transferase (GST) activity was found to be increased (11) in RA patients. But erythrocyte GR activity was reported to be increased in RA patients (23).

We found about 40% decrease in total protein SH levels in RA patients. Our results are compatible with the study of Miesel and Zuber who found 45-75% diminished SH status in RA patients (21). These decrease in SH levels could be explained by the detection of high xanthine oxidase (XOD) activity and oxyradical-producing XOP/acetaldehyde system (21). Another explanation of SH depletion could be the excretion of urinary thiol compounds. Those patients active in disease had been found to have significantly higher levels of urinary thioamine excretion (24).

Protein oxidation status in terms of protein carbonyls was not studied exensively before. In a studies of Mantle et al. and Chapman et al., carbonyl content of proteins found in synovial fluid were found to be increased (25,26). Plasma car-

Table 3. Multiple regression analysis for serum total protein SH, protein carbonyls and MDA by variables of clinical features and blood biochemistry of RA

	Total protein SH		Protein carbonyls		MDA	
Variable	**Coefficient (β)**	**p**	**Coefficient (β)**	**p**	**Coefficient (β)**	**p**
ESR	-0.151	0.645	-0.306	0.228	-0.096	0.786
CRP	0.416	0.114	-0.661	0.003	-0.013	0.862
RF	-0.030	0.918	0.052	0.816	-0.124	0.696
Age	-0.189	0.555	-0.001	0.997	-0.144	0.677
Disease.Duration	-0.072	0.772	0.080	0.672	0.084	0.755
N .Tender joints (0-28)	-0.035	0.895	0.040	0.845	0.256	0.383
N.Swollen joints (0-28)	-0.478	0.149	0.061	0.801	0.022	0.949

Total protein SH- $R^2=0.245$, $P=0.677$, Protein carbonyls- $R^2=0.568$, $p<0.05$, MDA- $R^2=0.117$, $P=0.951$

bonyl status was assessed only in juvenile chronic arthritis patients (JCA) and a correlation was found between carbonyl groups and the activity of JCA (27). Lipid peroxidation was also evaluated in RA patients. Increase in serum or synovial fluid MDA levels were reported in a several papers (28-34). Another lipid peroxidation product, conjugated dienes were also found to be increased in RA patients (35). Although, increased MDA levels has been found to be associated with RA in several papers, Kajanachumpol et al. found an unchanged MDA levels in RA patients (35). As we found, increase in protein carbonyl and MDA levels and decrease in SH status confirm the ROS mediated molecular changes in RA patients. Negative correlations were found between protein SH levels and MDA and protein carbonyl levels ($r=-0.416$, $p<0.05$ and $r=-0.465$, $p<0.05$) respectively. Only protein carbonyl levels were found to be negativelly correlated with ESR and CRP levels ($r=-0.415$, $p<0.05$ and $r=-0.687$, $p<0.001$) respectively. No correlation was found between RF values and different oxidative stress markers.

No correlation was found between different oxidative stress markers and biochemical markers in patients active in disease but negative correlations was found between carbonyl levels and ESR and CRP levels in patients in remission. Although this is a very small study group, this may be due to insufficient response to medical treatment is not enough to diminish the production of ROS and confirm the contribution of other sources for ROS production other than inflammatory response. During this period, protein oxidation continues either due to pre-formed radical mediated changes or other free radical producing sources. Decrease in SH levels and increase in MDA levels during active period in disease confirm the inflammation induced ROS production and consequently the ROS mediated changes in macromolecules. It was also concluded that, SH groups on proteins could be the first line antioxidants and lipids could be the one of the first macromolecules to be oxidized by ROS which is followed by proteins.

As a conclusion, ROS seems to play an important role in inflammatory response in RA. Measurement of serum total SH, protein carbonyl and MDA levels could be used as a supporting tests in RA and also could be used to discriminate RA patients whether they're active in disease or in remission. Although there's a slight increase in SH levels in RA patients in remission, new therapeautic approaches should be addressed especially to increase total protein SH levels in order to avoid further oxidative attacks.

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