Preliminary results regarding certain redox stress parameters and peripheral blood mononuclear cell membrane fluidity for rheumatoid arthritis patients

Received for publication, November, 10 2008
Accepted, may, 10 2009

NEGREI CAROLINA¹, MARGINA DENISA², BALANESCU ANDRA³, IULIE MIHĂELEA¹, GLIGA ANDA², BACONI DANIELA LUIZA¹, BALALAU DAN¹
¹Department of Toxicology, University of Medicine and Pharmacy “Carol Davila”, Bucharest, Romania
²Department of Biochemistry, University of Medicine and Pharmacy “Carol Davila”, Bucharest, Romania
³Research Center of Rheumatic Diseases, Sf. Maria Hospital, University of Medicine and Pharmacy Carol Davila, Bucharest, Romania
Corresponding author: Negrei Carolina, 6 Traian Vuia Str., Bucharest, phone/fax 0213111152, e-mail: carol_n2002@hotmail.com

Abstract

The present study presents preliminary results aimed to evaluate certain biochemical redox stress parameters (erythrocyte activity of glucose-6-dehydrogenase – G6PDH, erythrocytes susceptibility to lipid peroxidation – ESP) and peripheral blood mononuclear cells (PBMC) membrane fluidity at rheumatoid arthritis (RA) patients compared to healthy controls.

A clinical study was designed that included 36 subjects, (20 RA patients compared to a control group of 16 healthy subjects). On red blood cells separated from a jeun blood samples, G6PDH and ESP were assayed. PBMC were separated using the gradient density method and were spectrofluorimetrically evaluated for the membrane fluidity, by using TMA-DPH as a fluorescent probe.

The results showed that the RA is associated with a reduction of the G6PDH activity, and a subsequent decrease of the NADPH production (thus of the antioxidant defense system). The increase of the ESP is connected to the reduction of the liposoluble erythrocytes’ membrane antioxidant content.

The rheumatoid arthritis patients are also characterized by pathological changes of the PMBC membrane, pointed out by a lower TMA-DPH permeation of the cell membrane, which could be due to the immune activation of these cells and the expression of receptors on their surface.

Keywords: rheumatoid arthritis, redox stress, membrane fluidity

Abbreviations: RA = rheumatoid arthritis, ROS = reactive oxygen species, NADP = nicotinamide adenine dinucleotide phosphate, PBS = phoshat buffer saline, TMA-DPH = 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate, PBMC = peripheric blood mononuclear cells, G6PDH = glucose-6-phosphate dehydrogenase, ESP = erythrocytes susceptibility to peroxidation, MDA = malondialdehyde, DMSO = dimethyl sulfoxide.

Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory chronic disease, with unknown etiology and autoimmune pathogenic mechanisms [1, 2]. The disease occurs when the body’s immune function mistakenly attacks the synovium resulting in joint damage, ongoing pain, inflammation, loss of function and disability. Since RA is a systemic disease, the inflammation can affect other organs than the joints as well.
Abnormal blood antibodies can be found in patients with rheumatoid arthritis, e.g. the rheumatoid factor (which can be found in 80% of patients), citrulline antibody, the antinuclear antibody, etc. [3, 4]. Therefore an integrative approach of the pathogeny reveals the fact that RA is characterized by a pathological immune response, determined by an unknown antigen in a genetically predisposed host, which cannot be controlled by physiological adjusting mechanisms.

Even if the pathogenical mechanisms of RA are still unknown, the clinical as well as the molecular biology research lead to the identification of a complex built up of an immune system cellular subset, as well as surface cell markers and cellular products involved in the inflammatory process associated with the RA. Also, recent studies reported an oxidative impairment at the synovial fluid level of RA patients determined by an increase in the production of reactive oxygen species, correlated with the decrease of the endogenous antioxidant system activity [5, 6, 7, 8, 9, 10]. Reactive oxygen species (ROS) are produced by aerobic organisms in physiological conditions; these intermediary metabolic products induce pathological modifications only if they are no longer counteracted by endogenous antioxidants.

Plasma membrane fluidity is a biophysical parameter which can give valuable information on various substances traffic through the cell membrane and transport of medicines to the targeted organ or tissue [11, 12]. It can also describe the capacity of a damaged cell membrane to perform a normal function. Even if the information provided by this parameter is a global one, when associated with other biochemical and biophysical parameters it can facilitate the understanding of mechanisms underlying different processes that are happening at the cell membrane level.

The present paper aims at describing the status of certain RA patients in what concerns the ROS production and the peripheric blood mononuclear cells membrane fluidity as compared to normal subjects in an attempt to find correlations between the investigated parameters.

Materials and methods

Study design

We designed a clinical study that included 36 subjects divided into two groups:
- the study group of 20 rheumatoid arthritis (RA) patients, 20 to 70 year aged, 5 men and 15 women, diagnosed according to the 1987 revised criteria of the American College of Rheumatology; the patients were treated with a disease modifying antirheumatic drug (methotrexate or leflunomide), none received corticosteroids;
- the control group of 16 healthy subjects (3 men and 13 women).

Patients with severe renal, hepatic or hematological diseases, overt cardio-vascular disease or malignancy were excluded from the study.

Informed agreement of all the subjects included in the study was obtained, and the working protocol was approved by the Ethics Committee.

Reagents: sodium citrate, sodium chloride, triethanolamine chloride, nicotinamide adenine dinucleotide phosphate (NADP⁺), glucose-6-phosphate disodium salt, phosphate buffer saline (PBS), thiobarbituric acid, sodium azide, trichloracetic acid, Hystopaque 1077 – were purchased from Merck and Sigma, RPMI 1640 medium with sodium bicarbonate and L-glutamine was purchased from Biochrom AG, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluenesulfonate (TMA-DPH) was obtained from Molecular Probes.
Devices: Cary 100 BIO UV-VIS absorption spectrophotometer (Varian Inc.), equipped with a Peltier thermostated cell holder; LS50 B spectrofluorimeter (Perkin Elmer), equipped with thermostated cell holder, magnetic stirring and fluorescence polarization accessory.

Sample preparation
Venous *á jeun* blood samples were drawn from the patients on EDTA Na₂ as anticoagulant.

The separation of the cells was performed with Hystopaque 1077° by using the gradient density method; the peripheral blood mononuclear cell (PBMC) ring was collected and processed. The red blood cells were separated, washed twice with NaCl 0.9% solution and standardized at 1g hemoglobin / 100 mL erythrocyte suspension. The erythrocyte glucose-6-phosphate dehydrogenase (G6PDH) activity was evaluated on freshly prepared hemolysate (1:3, v/v).

The PBMC cell ring obtained was twice washed with RPMI 1640 medium and standardized at 10⁵ cells/mL in RPMI 1640 medium.

Biochemical evaluation
The activity of G6PDH (EC 1.1.1.49) was assayed by the method of Lohr and Waller [13]. The rate of NADPH formation, which is a measure of the enzyme activity, was evaluated spectrophotometrically at 340 nm [14].

The red blood cells susceptibility to lipid peroxidation (ESP) was evaluated by means of the malondialdehyde (MDA) content on the standardized erythrocyte suspension [15, 16]. For this purpose, the erythrocyte suspension was treated for 1 hour with 10 mM H₂O₂ (1:1, v/v). The MDA resulted from the red blood cell membrane lipids peroxidation was evaluated spectrophotometrically at 535 nm by using thiobarbituric acid (1%, 3:0.75 v/v).

Membrane anisotropy evaluation
The membrane anisotropy of PBMC was assayed by the determination of TMA-DPH steady state fluorescence polarization after the exterior phospholipid layer permeation of the cell membrane.

The emission intensity of the normalized PBMC suspension was measured for 4 seconds every 0.02 seconds at 340 nm excitation and 425 nm emission wavelengths, with the polarizers oriented in the four possible positions (in the excitation and emission beams, respectively): vertical-vertical, vertical-horizontal, horizontal-vertical and horizontal-horizontal, thus generating the subsequent emission intensities: \( I_{0vv}, I_{0vh}, I_{0hh}, I_{0hv} \). To the same suspension, an aliquot of TMA-DPH stock solution in DMSO (2.5 µM TMA-DPH in cuvette) was added, the suspension with the fluorescent probe was incubated for 2 minutes at 37° C under continuous magnetically stirring, than the four resulting emission intensities were measured (\( I_{DPH_{vv}}, I_{DPH_{vh}}, I_{DPH_{hh}} \) and \( I_{DPH_{hv}} \)).

Calculation of the fluorescence anisotropy \( (r) \) was performed according to the equations (1) and (2):

\[
\begin{align*}
    r &= \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} \\
    G &= \frac{I_{hh}}{I_{vh}}
\end{align*}
\]

where \( I_{vv} = I_{DPH_{vv}} - I_{0vv}, I_{vh} = I_{DPH_{vh}} - I_{0vh}, I_{vv} = I_{DPH_{hh}} - I_{0hh} \) and \( I_{vv} = I_{DPH_{hv}} - I_{0hv} \).
Preliminary results regarding certain redox stress parameters and peripheral blood mononuclear cell membrane fluidity for rheumatoid arthritis patients

The membrane fluidity in the polar head-group region of the plasma membrane bilayer, $f$, was computed following eq. (3), as a function of limiting initial $r_0$ and long-time $r_\infty$ values of TMA-DPH fluorescence anisotropy [17, 18]:

$$f = \frac{r_0}{r_\infty}$$  \hspace{1cm} (3)

where $r_\infty = 1.270\times r - 0.076$ for $0<r<0.28$ and $r_\infty = 1.100\times r - 0.032$ for $0.28<r<0.34$ and $r_0=0.362$ [19].

As for part of the RA patients the membrane fluidity could not be computed, a binding parameter for the TMA-DPH to the cell membrane was evaluated by using eq. (4):

$$P = \frac{I_{DPH,vv} - I_{0,vv}}{I_{0,vv}}$$  \hspace{1cm} (4)

The results are presented as means, the statistical significance of the parameters was evaluated by means of Student’s t test.

**Results**

The results obtained for the investigated parameters are presented in Table 1.

<table>
<thead>
<tr>
<th>Evaluated parameters</th>
<th>RA patients</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Average</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>G6PDH (UI/g)</td>
<td>25.75</td>
<td>13.87</td>
</tr>
<tr>
<td>MDA (mM MDA/g Hb)</td>
<td>425.25</td>
<td>125</td>
</tr>
<tr>
<td>Membrane fluidity ($f$)</td>
<td>1.156</td>
<td>0.1032</td>
</tr>
<tr>
<td>Membrane binding parameter ($P$)</td>
<td>96.1</td>
<td>25.1</td>
</tr>
</tbody>
</table>

Table 1. Values of the biochemical redox stress parameters and peripheral blood mononuclear cell membrane biophysical parameters evaluated

According to literature, rheumatoid arthritis (RA) is generally associated with an increased redox stress. Our results confirm that, since G6PDH was significantly decreased 25.75±13.87 UI/g Hb vs. 190.45±27.89 UI/g Hb (p<0.0001) at RA patients compared to controls (Figure 1). The ESP was significantly increased (425.25±125.20 mM MDA/g Hb vs. 166.86±35.56 mM MDA/g Hb), p=0.0002 at RA patients compared to controls (Figure 1), thus indicating an overproduction of ROS.

![Figure 1. Redox stress evaluation of RA patients as compared to controls](image_url)
The evaluation of the membrane fluidity in the polar head group of the PBMC’s belonging to RA patients and the control group resulted in comparable mean values (1.156±0.010 vs. 1.176±0.018, p=0.6615). For part of the RA patients, the anisotropy of the TMA-DPH was either negative (the fluorescence intensity with the probe was lower than without it) or exceeded the maximum value of the probe (0.352), hence and a fluidity order parameter could not be computed. This kind of situation appeared more frequent at the RA patients (50%) than in controls (10%), suggesting that the outer layer of the cell membrane may be affected in the RA patients, making difficult to impossible the insertion of the fluorescent probe in the lipid bilayer of the membrane.

In order to quantify this effect, we computed a binding (permeation) parameter following eq. (4), and found that the mean value for RA patients is different from that one of controls (96.1±25.1 versus 392.1±68.6 – Figure 2).

![Figure 2. Membrane fluidity (f) and membrane permeability factor to TMA-DPH (P)](image)

**Discussions**

Rheumatoid arthritis (RA) involves two basic mechanisms of ROS production: activated polymorphonuclear cells (PMNs) and injury resulting from ischemia and reperfusion in the inflamed joints. These reactive species, if not scavenged, lead to lipid peroxidation [20].

Circulating human erythrocytes possess the ability to scavenge ROS generated extracellularly by activated neutrophils, by superoxide dysmutase, catalase and GSH-dependent mechanisms. The increase of the ESP at RA patients illustrates the reduction of the antioxidant systems and the increase of the ROS action at the erythrocytes’ level.

Our results show that the RA is associated with a reduction of the G6PDH activity, and a subsequent decrease of the NADPH production and of the antioxidant defense. The results are in agreement with literature data stating that joint diseases and RA in particular are associated with oxidative stress, due to the overproduction of ROS, and also to the effect of TNFα, that diminishes the activity of SOD and other antioxidant enzymes. Thus, medications
Preliminary results regarding certain redox stress parameters and peripheral blood mononuclear cell membrane fluidity for rheumatoid arthritis patients

that target ROS-induced damage to joint cartilage may deserve to be included in therapeutic strategies designed to combat the development and progression of inflammatory RA [21].

The rheumatoid arthritis patients are also characterized by pathological changes of the PMBC cell membrane, which could be determined by the immune activation of these cells and the expression of receptors on their surface; the changes are suggested by the lower binding (inside permeation) of TMA-DPH to the cell membrane of RA patients compared to controls.

Studies performed by Beccerica [22] show that there is a tendency of membrane rigidization in RA patients. Our results confirm this tendency (the membrane fluidity is slightly lower in RA patients compared to controls, but the differences are not statistically relevant within the probability of 0.05). However, significant differences were obtained between groups in what concerns the ability of TMA-DPH to insert within the membrane bilayer, pointed out by means of parameter P, which has significantly lower values for RA patients compared to controls, suggesting structural and/or functional changes of the PBMC’s from RA patients.

Conclusions

The redox stress status of RA patients was compared to healthy controls by means of G6PDH activity and ESP. The results indicate an increase of the ESP at RA patients by the increase of the ROS action at the erythrocytes’ level and also a reduction of the G6PDH activity, and a subsequent decrease of the NADPH production and of the antioxidant defense.

The membrane fluidity of PBMC was not different in RA patients compared to controls; however, for 50% of the patients (compared to 10% of the healthy controls) the membrane fluidity could not be computed due to the poor binding of the probe (TMA-DPH) to the polar head group of the phospholipids, suggesting changes or impaired function at the plasma cell membrane level.

Acknowledgements

The work was supported by grant TD 126/01.10.2008 of the National University Research Council.

References

[6]. Y OZKAN, S YARDIM-AKAYDIN, A SEPIC et al., Clin Rheumatol, 26, 64–68 (2007)
[7]. S TAYSI, F POLAT, M GUL et al., Rheumatol Int 21, 200–204 (2002)