Estrogen receptors, ERK 1/2 phosphorylation and reactive oxidizing species in red blood cells from patients with rheumatoid arthritis

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Abstract

Background: Rheumatoid arthritis (RA) is a chronic autoimmune disease associated with a significantly increased risk of cardiovascular mortality, mainly attributed to accelerated atherosclerosis.

Methods: Thirty-two women (aged more than 18 years) with RA, and 25 age-matched healthy women were included in this study. Biomarkers of inflammation, red blood cells (RBCs) redox balance, estrogen receptor alpha (ER-α) expression as well as ERK 1/2 phosphorylation content were evaluated in RA patients at baseline and six months after treatment with disease modifying anti-rheumatic drugs (DMARDs).

Results: For the first times we demonstrated that in RA patients: i) disease activity score (DAS-28) positively correlated with RBC ER-α expression, and negatively with total antioxidant capacity of plasma; ii) RBC ER-α expression positively correlated with systemic inflammatory biomarkers and oxidative stress parameters as well as ERK 1/2 phosphorylation; and iii) DMARDs treatments improved the clinical condition measured by DAS-28 score decrease, although the RBCs appeared to be more prone to pro-oxidant status associated to the expression of survival molecules.

Conclusion: Our data strongly suggest that RBCs could also participate in vascular homeostasis through fine modulation of an intracellular signal linked to the ER-α.

Keywords: rheumatoid arthritis; inflammation, oxidative stress; red blood cells; estrogen receptors.
1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by a strong systemic inflammatory condition that accelerates development of atherosclerosis caused by the inflammation-mediated endothelial dysfunction and vascular damage in the blood vessels.

Compared to men, women are 2 to 5 times more likely to develop RA. They have a high prevalence of cardiovascular disease (CVD), hypercoagulability, and consequently increased risk of developing cardiovascular events such as stroke and myocardial infarction [1-4].

A common feature of autoimmune inflammatory diseases, including RA, is the increased formation of reactive oxygen species (ROS), reactive nitrogen species (RNS), and chloride-derived species produced by activated immune cells in inflamed tissues [4-5]. In particular, for RA inflammation-induced oxidative stress is widely recognized to be key player in the pathogenesis of the disease [3-4,6-8]. The infiltration of immune cells into the synovial joint lining, the release of the pro-inflammatory cytokines, the redox imbalance linked to the release of oxidizing species by activated cells and the following alteration of intracellular signaling, as well as the impairment of the endogenous antioxidant system, are responsible for the oxidative damage and destruction of joints ultimately leading to substantial disability in RA patients [3,9-10]. A positively correlations between ROS and RNS with inflammation and accelerated joint destruction has been reported [3]. ROS (O$_2^-$ and H$_2$O$_2$) and RNS (NO and its derived oxidants) have indeed reported to be generated in the inflamed joint of RA patients by chondrocytes, activated macrophages in the synovial membrane or by the activated neutrophils in the synovial cavity, both in the cytosol and in the mitochondria leading to oxidative damage of the cartilage, the extracellular matrix, collagen and proteoglycans [6]. Furthermore, ROS and RNS are known to induce lipid peroxidation that yields to the formation of
lipid hydroperoxides and electrophilic reactive lipid species end products, including malondialdehyde (MDA), which can form adducts with proteins able to modify their activity and function [8].

Under physiological conditions, besides their role as oxygen/carbon dioxide carriers, red blood cells (RBCs) play a key role in maintaining vascular homeostasis. These cells are excellent scavengers of ROS and RNS being equipped with an efficient antioxidant system able to maintain the balance between the blood anti-oxidant and pro-oxidant status as well as to control the vasodilatation through the transport and release of NO [11]. Under pathophysiological conditions, changes in the redox state of RBCs shift their role from antioxidant defense to pro-oxidant state. This can induce dysfunction and progression of CVD [11].

In a previous pilot study performed by our group, in view of their activity as redox effectors or “scavengers” as well as determinants in thrombus formation, we evaluated the redox state and lifespan molecules of RBCs isolated from RA patients investigating whether they were conditioned by systemic oxidative stress [12]. In this study we found that, compared to healthy donors (HD), plasma from RA patients showed increased MDA levels and decreased total antioxidant capacity (TAC) suggesting the occurrence of systemic oxidative stress as also reported in previous reports by other groups [13-15]. In addition, we found that this systemic oxidative imbalance did not seem to significantly interfere with the redox state of RBCs. In fact, compared to HD, in RBCs from RA patients, a mild increase of ROS/RNS level as well as a mild reduction of total thiol content was detected [12]. Moreover, no differences in the percentage of RBCs undergoing eryptosis, the programmed death of injured RBCs, were detected. Conversely, these cells showed a significant up-regulation of survival molecules, such as survivin, as well as increased levels of the phosphorylated mitogen-activated protein kinase ERK 1/2 [12]. In addition, we recently demonstrated that estrogen receptors (ER-α and ER-β): i) were expressed by RBCs; ii) were both functionally active, iii) played a role in the modulation of RBCs intracellular signaling, and iv) modulated RBCs eNOS activation and NO release [16]. These data strongly suggested that RBCs could participate to vascular homeostasis also through a fine modulation of an estrogen receptor-linked intracellular signaling and
redox modulation. On the basis of these observations, we evaluated the ERs expression in RBCs from RA patients and their correlation with the activity of disease, with levels of ERK 1/2 phosphorylation and with inflammation- and oxidative stress-related biomarkers before and after six months of treatment with appropriate disease modifying anti-rheumatic drugs (DMARDs).
2. Materials and Methods

2.1. Patients

Thirty-two women (aged more than 18 years) affected by RA diagnosed according to 2010 ACR (American College of Rheumatology) criteria, coming from Early Arthritis Clinic and 25 age-matched healthy women (HD), were recruited at the Rheumatology Unit of Sapienza University of Rome. The study was reviewed and approved by the Local Ethical Committee at the Sapienza University. A written informed consent was obtained from all patients. All clinical investigations have been conducted according to the principles expressed in the Declaration of laboratory data to measure the Disease Activity Score (DAS-28), which takes into account the number of swollen and painful joints (out of 28 joints), acute-phase reactants (ESR, CRP), and patient's global health. Smokers and patients with hypercholesterolemia, arterial hypertension, cardiovascular diseases, type 1 and 2 diabetes and cancer were excluded from the study.

In particular, two groups of RA patients were evaluated: early- and long-standing disease patients. The first group of patients was enrolled before the treatment, while the second had been treated with methotrexate and did not responded to therapy. All patients were assessed at baseline and after six months (follow up) of treatment with the DMARDs methotrexate and anti-TNF-α, respectively.

2.1. Red blood cells isolation

Fresh human blood from healthy donors was drawn into heparinized tubes. For RBCs isolation, whole blood was centrifuged for 10min at 1.500g. The plasma and buffy coat were removed. RBCs were washed twice in isotonic PBS, pH 7.4, and suspended in the same buffer to the initial hematocrit concentration. No appreciable cell lysis was observed during the RBC preparation procedure.
2.2. Red blood cells redox balance

To evaluate the formation of total intracellular reactive oxidizing species, RBCs (5x10^5 cells) were incubated in the Hanks’ balanced salt solution, pH 7.4, containing dihydrorhodamine 123 (DHR123; Molecular Probes). Samples were then analyzed with a fluorescence-activated cell-sorting (FACS) flow cytometer (Becton Dickinson, Mountain View, CA, USA). The median values of fluorescence intensity histograms were used to provide semiquantitative evaluation of the oxidizing species production.

2.3. Analytical cytology

RBCs were fixed with 3.7% formaldehyde in PBS (pH 7.4) for 10 min at room temperature, washed in the same buffer and permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature. After washing with PBS, samples were incubated for 30 minutes at 37°C with monoclonal antibodies: anti-ER-α, anti-ER-β, anti-phosphorylated ERK 1/2 (BD PharMingen, San Diego, CA), anti-survivin (Santa Cruz Biotechnology) and anti-3-nitrotyrosine (Sigma Aldrich). After all, samples were washed thrice in PBS to be then incubated with secondary antibody FITC-conjugated: anti-mouse (Invitrogen, Carlsbad, CA) or anti-rabbit (Invitrogen, Carlsbad, CA). All the samples were recorded with a FACScan flow cytometer (Becton-Dickinson, Mountain View, CA, USA) equipped with a 488nm argon laser. At least 20,000 events were acquired. The median values of fluorescence intensity histograms were used to provide a semi-quantitative analysis.

2.4. Evaluation of plasma total antioxidant capacity (TAC)

To evaluate TAC of plasma a commercially colorimetric assay kit has been used (BioVision, abca company).
2.6. Statistical analyses

Cytofluorimetric results were statistically analyzed by using the nonparametric Kolmogorov–Smirnov test using Cell Quest Software. At least 20,000 events were acquired. The median values of fluorescence intensity histograms were used to provide a semi-quantitative analysis. Student’s t-test was used for the statistical analysis of the collected data. Correlations were evaluated by using Pearson correlation (r correlation coefficient). To test the probability of significant differences, individual group comparisons were evaluated using Bonferroni’s test. p \leq 0.05 values were considered statistically significant.
3. Results

3.1. Patient profile

In this study, two different groups of female RA patients comparable for age and showing different duration of the disease: early- and long-standing disease, have been investigated. As shown in Table 1, the disease duration was 5.5 times lower in early-standing RA patients with respect to long-standing patients. When compared for the DAS-28 score, the early-standing patients showed high (71% of patients) and moderate (29% of patients) disease activity (DAS-28: 4.47 ± 1), while the long-standing patients showed low (35.7% of patients) and moderate (64.3% of patients) disease activity (DAS-28: 3.8 ± 0.8). The disease activity was sustained in early-patients by the increased concentration of the acute-phase biomarkers, such as rheumatoid factor (RF), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), whose values were significantly higher (p ≤ 0.05) than in long-standing patients.

| Table 1. Clinical characteristics of RA patients |
|-----------------------------------------------|
| **Early disease** (n = 19) | **Long standing disease** (n = 13) | **P values** |
| **Age (y)** | Median 52 (range 19-75) | Median 49 (range 19-77) |  |
| **Sex** | Female | Female |  |
| **Disease duration (weeks)** | Median 21.5 weeks (range 3-52) | Median 118 weeks (range 54-324) | 1.48 |
| **DAS 28 score** | 4.47 ± 1 | 3.8 ± 0.8 | 0.166 |
| **RF (IU/ml)** | Median 183 (range 10 – 600) | Median 28 (range 8-137) | 0.043* |
| **CRP (mg/l)** | Median 12.88 (range 0.4 – 56) | Median 3.84 (range 0.1 – 15.8) | 0.05* |
| **ESR (mm/h)** | Median 33.5 (range 4 – 76) | Median 14 (range 4 – 25) | 0.012* |

RA = rheumatoid arthritis; DAS 28 score = disease activity score; RF = Rheumatoid factor; CRP = C reactive protein; ESR = erythrocyte sedimentation rate.
3.2. Estrogen receptors expression and localization in RBCs from RA patients

By using flow and static cytometry we therefore evaluated ER-α and ER-β expression and localization in RBCs from HD, early- and long-standing RA patients. As shown in the Figure 1A, in comparison with HD, the semi-quantitative cytometric analysis showed that ER-α expression was: i) increased, but not significantly, in RBCs from early- patients; and ii) similar in RBCs from long-standing patients. Interestingly, a significant (p < 0.05) difference in ER-α expression was detected between RBCs from early- and long-standing RA patients.

With regard to ER-β expression, no significant differences were detected in RBCs from HD and RA patients (Figure 1C). Typical flow cytometric profiles of ER-α and ER-β content in RBCs from a representative HD, a representative early RA patient and a long-standing RA patient were shown in the Figure 1B and 1D. Moreover, by static cytometry we found that both ER-α and ER-β were mainly localized in the RBC membrane. Micrographs obtained by static cytometry showing ER-α and ER-β distribution in RBCs from a representative HD, a representative early RA patient and a long-standing RA patient are shown in Figure 1E.
Fig. 1
3.3. Correlation between disease activity, oxidative stress and ER-α expression

As expected, DAS-28 score negatively correlated with plasma TAC (r = -0.71). Surprisingly, a positive correlation was found between DAS28 score and ER-α content (r = 0.22). These results suggested that the expression of ER-α on the RBCs membrane of RA patients might be linked to the exogenous inflammatory and oxidative stress conditions and that these cells, due to the link between the estrogen receptor expression, the activation of intracellular signaling and the redox modulation [12,16], may become in turn source of reactive oxidizing species.

3.4. Correlations between the ER-α expression and the parameters linked to inflammation and oxidative stress

To evaluate a likely association between the ER expression and the parameters linked to the inflammation and oxidative stress, we correlated the ER-α expression with the acute-phase and the oxidative stress biomarkers, as well as with the expression of the phosphorylated form of ERK 1/2, taken as a marker of the kinase activation (Table 2). We found that ER-α expression in RBCs from RA patients negatively correlated with plasma TAC and positively correlated with i) the inflammatory biomarkers, i.e., ESR and CRP), ii) the oxidative stress-related biomarkers, i.e. ROS/RNS and 3-nitrotyrosine (3-NT), and iii) the p-ERK 1/2 expression (Table 2).
Table 2. Correlation of ER-α with various selected variables

| Variables     | r     | p values |
|---------------|-------|----------|
| TAC           | -0.050 | 0.884    |
| ESR           | 0.116  | 0.719    |
| CRP           | 0.280  | 0.402    |
| ROS/RNS       | 0.354  | 0.258    |
| 3-NT          | 0.545  | 0.066    |
| p-ERK 1/2     | 0.550  | 0.124    |

TAC = total antioxidant capacity of plasma; ESR = erythrocyte sedimentation rate; CRP = C reactive protein; ROS = reactive oxygen species; RNS = reactive nitrogen species; 3-NT = 3-nitrotyrosine; p-ERK ½ = phosphorylated extracellular signal-regulated kinase; TAC = total antioxidant capacity of plasma.

3.5. Patient treatment and follow up

As required by the therapeutic treatment for RA, early and long-standing RA patients were treated with the DMARDs methotrexate and anti-TNF-α, respectively, and monitored after a 6 months long survey. As expected, comparing clinical characteristic acquired at the patients recruitment and reported in Table 1, after six months of treatment, the disease activity was significantly reduced in methotrexate- (DAS-28: 3.2 ± 1.3) and anti-TNF-α-treated (DAS-28: 2.9 ± 05) in both early and long-standing patients, respectively. Furthermore, in both group of RA patients DMARDs treatment increased the expression of ER-α, p-ERK, as well as ROS/RNS and 3-NT (Table 3). Interestingly, the treatment also strongly increased, in both patients groups, the expression of RBC survivin, a survival molecule that, in a preliminary study we found increased in RBCs from RA patients with respect to HD [12].
### Table 3. Expression of some variables 6 months after therapy (F.U.)

| Variables | Early standing RA F.U. | Long standing RA F.U. |
|-----------|------------------------|-----------------------|
| ER-α      | Δ% + 8                 | Δ% + 188              |
| p-ERK 1/2 | Δ% + 55                | Δ% + 67               |
| Survivin  | Δ% + 145               | Δ% + 122              |
| ROS/RNS   | Δ% + 16                | Δ% + 10               |
| 3-NT      | Δ% + 32                | Δ% + 67               |

### 4. Discussion and conclusion

In this work, we showed for the first time that in RA patients: i) DAS-28 score positively correlated with RBC ER-α expression; and ii) the treatment with DMARDs, if on the one hand improved the clinical condition measured by the reduction of DAS-28 score, on the other hand increased the oxidative stress-related biomarkers and the expression of survival-related proteins [3,7,13]. In addition, our data also confirmed that DAS28 score negatively correlated with TAC [13-15].

Sex hormones and their receptors in autoimmune diseases have been previously investigated and both ER-α and ER-β have been identified in different types of synovial cells from RA patients with their expression being up-regulate by some inflammatory factors [17]. In this work we found that both ER-α and ER-β were expressed in RBCs from RA patients, but only ER-α content was slightly increased in early diseased RA patients with respect to HD and long-standing RA patients. The positive correlation between the DAS-28 score and the RBCs ER-α expression associated the disease activity to the estrogen receptor-mediated activation of a signaling pathway linked to the occurrence of mild oxidative stress and to the alteration of redox state in diseased RBCs [16]. Moreover, the positive association between the ER-α expression, ERK 1/2 phosphorylation and ROS/RNS production in RBCs further supported the hypothesis of involvement of a kinase-linked intracellular signaling whose activation, downstream to estrogen receptor, could play a key role in the induction of the mild oxidative stress detected in RBCs from RA patients [16]. Similarly to what detected in
RBCs from HD in this and in our previous work [16], the immunofluorescence study showed that in RBCs from RA patients, ER-α was mainly localized at the plasma membrane and its content was slightly, although not significantly, higher in early- with respect to long-standing RA patients and HD. The membrane-bound ER-α has been reported to play a critical role in RBCs homeostasis by stimulating the intracellular signaling linked to the kinase pathways [16]. The activation of this non-genomic pathway, occurring in cells deprived of the nucleus such as in RBCs, involves several important kinases, including the mitogen-activated protein kinases (MAPK) ERK 1/2, AKT, and P38 [18-20]. In particular, ERK 1/2 regulates critical cellular activities, such as cell adhesion and cell survival [21] and the level of its phosphorylation has been found higher in RBCs from healthy females with respect to males [16]. RBCs from RA patients have been shown to undergo to a mild alteration of intracellular redox state associated to the activation of the signaling linked to ERK 1/2, whose phosphorylation induced the up-regulation of the apoptotic inhibitor survivin, which, in turn, is recognized to favor the RBC aggregation and adhesion to the endothelium other blood cells [16,22].

In addition, the membrane-associated ER-α has been reported to mediate the endothelial NOS phosphorylation and the NO production through a mechanism involving the activation of several kinases, including c-Src, phosphatidylinositol 3-Kinase and Akt [23-24]. It has been reported that in RBCs, the activation of the NOS endothelial isoform and the production of NO were triggered by ERK 1/2 phosphorylation dependent on ER-α stimulation [16]. Notably, in RA patients, the link between the ER-α and the NO pathway was further suggested by the positive correlation between ER-α expression and 3-NT amount in RBCs. The formation of 3-NT is considered the footprint of RNS formation with particular regards to peroxynitrite, the product of the fast reaction between O2•− and NO, and nitrite-derived nitrating species. In autoimmune diseases, tyrosine nitrated proteins have been hypothesized to behave as neoantigens boosting the formation of autoantibodies [25]. The formation of peroxynitrite inside RBCs from RA patients is conceivable as a result of the increased: i) NO synthesis by the eNOS stimulated by ER-α activation and ii) O2•− formed as a result of hemoglobin auto-oxidation and activation of the NADH oxidases following the RBCs continuous
exposition to exogenous oxidative insult [11,26]. Interestingly, both DAS-28 score and ER-α expression in RBCs of RA patients negatively correlated with plasma TAC. It is expectable that the severity of the disease could be associated to a decreased TAC, just because oxidative stress plays a key role in RA pathogenesis also inducing the depletion of the endogenous antioxidant defenses linked to the continuous detoxification of the oxidizing species released from inflamed diseased tissues. Similarly, the negative correlation between ER-α expression and TAC indirectly links the increase of oxidative stress and the RBCs activation mediated by the estrogen receptor.

As mentioned above, under condition of increased systemic oxidative stress that occurring in tissues of RA patients [3,9-10], an RBC pro-oxidant status could be favored by the alteration of intracellular redox state and the decrease of the intracellular antioxidant defense, as well as by the increase or intracellular oxidative milieu connected to the activation of the ER-α pathway [16]. Several studies have shown positive correlation between the disease severity and the parameters linked to oxidative stress in RA patients reinforcing the association between oxidative/nitrosative stress and the disease. In particular, DAS-28 of RA patients has been positively correlated with the levels of lipid peroxidation products such as the serum levels of MDA [3,9,27], with the formation of oxidizing species such as O₂⁻, H₂O₂, ·OH and NO [3,9-10], as well as the MPO-derived hypochlorous acid [28] with protein oxidation [3] as well as with the low molecular weight antioxidant system impairment such as the decrease of the levels of vitamins C and E, glutathione, β-carotene, zinc and selenium [3]. High molecular weight antioxidants showed contrasting correlation with the disease activity being increased, decreased and even unaltered levels, such as in the case of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase [6]. The positive correlation between RBC ER-α expression and the oxidative stress biomarkers, as well as its negative correlation with TAC support a role of the estrogen receptor-linked pathway in the activation of RBCs of RA patients.

The treatment of RA patients with the DMARDs methotrexate and anti-TNF-α prescribed to early and long-standing RA patients, respectively, increased RBC ER-α expression, p-ERK 1/2, the
oxidative stress related parameters as well as the expression of the content of survivin, an inhibitor of the apoptotic pathway. It has been reported that the pro-oxidant effects induced by methotrexate are due to reactive oxidizing species generation, antioxidant enzyme activity inhibition and decrease of low-molecular weight antioxidants [5]. Moreover, differences in the clinical effectiveness of the therapy with anti-TNF-α, has been found in RA patients, with particular regard to non-responder patients in which the circulating inflammatory profile and the oxidative stress biomarkers remained significantly elevated after 6 months of therapy [29].

Although the patients DAS-28 score was decreased and indicated an improvement of patient clinical condition confirming the beneficial effects of the treatment, the increase of the oxidative stress-related parameters linked to ER-α suggests that RBCs from treated patients are more prone to pro-oxidant status associated to the expression of survival molecules, such as survivin. This molecule has been shown to play a critical role in RA conferring a long-lasting feature to immune cells through apoptosis inhibition [30]. In addition, survivin has been found increased in RBCs form RA patients [16], as well as in RA synovial tissues [31] and positively correlated with DAS-28 score [31]. Its increased expression could indicate that, similarly to what reported for lymphocytes and fibroblast-like synoviocytes in joints of RA patients [30], also RA RBCs survive for a long time as a consequence of compromised apoptosis. Since our results show that the activation of ERK 1/2 is linked to the expression of ER-α in RBCs from RA patients, the kinase phosphorylation could induce the upregulation of survivin and the increase of diseased RBC adhesion to the endothelium [22].
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**Figure legend**

**Figure 1. Expression and localization of ERs in RBCs**

Flow cytometry analysis of: (A) ER-α expression in RBCs from HD, early- and long-standing RA patients; (B) ER-α expression in RBCs from a representative HD, a representative early standing RA patient and a representative long-standing RA patient; (C) ER-β expression in RBCs from HD, early- and long-standing RA patients; (D) ER-β expression in RBCs from a representative HD, a representative early standing RA patient and a representative long-standing RA patient. (E) Micrographs obtained by static cytometry showing ER-α and ER-β distribution in RBCs from a representative HD, a representative early standing RA patient and a long standing RA patient.