

Report

Band 3 Protein Function and Oxidative Stress in Erythrocytes from Systemic Sclerosis Patients with Interstitial Lung Disease

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Abstract: A link between reactive oxygen species (ROS) and Systemic Sclerosis (SSc) development has been already described, along with an oxidative stress-induced reduction of anion exchange capability through Band 3 protein. On this basis, blood from SSc patients has been collected to measure the rate constant for SO_4^- uptake, which is an index for anion exchange capability through Band 3 protein. This parameter was significantly reduced in SSc patients, with or without Interstitial Lung Disease, a complication of SSc. As a reduced Band 3 protein function was observed in SSc patients with either low or high levels of malondialdehyde (MDA), the end product of lipoperoxidation, anion exchange through erythrocytes membrane may be not univocally related to oxidative stress and, what is more, can be considered as a sensitive tool to monitor SSc development.

Keywords: Band 3 Protein, Erythrocytes, Interstitial Lung Disease, Oxidative Stress, Systemic Sclerosis

1. Introduction

The role of oxidative stress in pathogenesis diseases has risen much more attention in the recent years, with specific regard to diabetes [1], aged-related pathologies including neurodegenerative diseases [2-4] and, most recently, canine leishmaniasis [5]. Systemic Sclerosis (SSc), an autoimmune connective tissue disorder, has been chosen as subject of the present report. SSc is associated with many different clinical patterns, at tissue and internal organ levels, possibly mediated by progressive fibrosis. Skin lesions and microvascular alterations are also described. Though its pathophysiology is still unclear, a link between reactive oxygen species (ROS)

and SSc development has been ascertained [6]. In particular, high ROS levels in patients with diffuse cutaneous SSc (dcSSc) have been associated with increased levels of oxidative stress markers, alteration of antioxidant mechanisms, reduced non-enzymatic antioxidants, such as ascorbic acid, α -tocopherol, β -carotene and selenium and reduced antioxidant defense [7-10]. The link between oxidative stress and SSc has been further supported by a mouse model exhibiting a disease closely resembling human SSc when administered hypochlorous acid, as oxidative agent [11].

This background motivated the focus on SSc and its complications and led to monitor Band 3 protein efficiency in erythrocytes of SSc patients displaying pulmonary fibrosis as a complication of such disease.

Band 3 protein is the most abundant integral membrane protein of erythrocytes [12, 13], responsible for $\text{Cl}^-/\text{HCO}_3^-$ exchange, which correlates with ion balance across membrane, osmotic properties, cell shape maintaining, gas exchange and, in turn, oxygenation of the entire organism [14]. These functions are mediated by both membrane and cytoplasmic domains *via* phosphorylation pathways, as recently shown [15, 16]. Other studies have demonstrated a reversible interaction of Band 3 protein with hemoglobin and spectrin [17, 18], suggesting that the efficiency of Band 3 protein reflects whole erythrocyte homeostasis.

$\text{SO}_4^{=}$ transport measurement, as more easily detected than Cl^- or HCO_3^- uptake [12], significantly accounts for $\text{Cl}^-/\text{HCO}_3^-$ exchange and can be monitored by a turbidimetric method [12-19] based on measurement of the rate constant for $\text{SO}_4^{=}$ uptake. This method, enabling determination of anion exchange velocity, has been recently confirmed as a good tool to verify the impact of oxidative stress on human erythrocytes exposed to xenobiotics, natural toxins or hydrogen peroxide [19, 20]. With regard to this latter point, the authors [19] demonstrated that ion transport through erythrocytes membrane is a sensitive tool to detect oxidant effects provoked by hydrogen peroxide at concentrations within a physiological range (under 1 mM) and not eliciting production of Malondialdehyde (MDA). MDA is the end product of lipid peroxidation, and hence considered as an index of lipoperoxidation.

Based on this evidence, the aim of the present report was to pursue this novel issue in rheumatology by checking anion exchange capability through Band 3 protein in erythrocytes from SSc patients with pulmonary fibrosis, recruited in Southern Italy (Sicily). Patients gave their informed consent prior to their inclusion in the protocol which was carried out in accordance with the Declaration of Helsinki and Good Clinical Practice.

Blood samples were withdrawn from 15 healthy patients and 8 SSc patients with no HRTC (High resolution CT) sign of pulmonary fibrosis. Nine SSc patients (6 females and 3 males) were affected by interstitial lung disease (SSc-ILD). SSc patients with no HRTC signs of pulmonary fibrosis had 51.2 ± 9.4 years of age, while those with ILD had 53.2 ± 7.6 years of age. All patients were affected by the diffuse form of disease. Disease duration, considered as the time since the first non-Raynaud symptom, was 3.5 ± 2 years for patients with ILD and 4.1 ± 2.3 for SSc patients with no HRTC sign of pulmonary fibrosis.

Erythrocytes were suspended to 3% hematocrit in isotonic medium, containing 116 mM Na_2SO_4 , 10 mM HEPES, 15 mM glucose, pH 7.4, osmotic pressure 300 mOsm. Levels of $\text{SO}_4^{=}$ were spectrophotometrically read at 425 nm wavelength, and the absorption was converted to millimolar of intracellular $\text{SO}_4^{=}$ using a calibrated standard curve previously obtained by precipitating known $\text{SO}_4^{=}$ concentrations (mg/L cells 10^{-2}). [$\text{SO}_4^{=}$] L cells 10^{-2} reported in graphs represent $\text{SO}_4^{=}$ concentration trapped by 10 mL erythrocytes suspended at 3% hematocrit. Intracellular $\text{SO}_4^{=}$, determined at different time intervals (5-10-15-30-45-60-90-120 min), was considered to

calculate the rate constant (min^{-1}) [18]. Reciprocal of the rate constant for $\text{SO}_4^{=}$ uptake (min) represents time needed to reach 63% of the total $\text{SO}_4^{=}$ intracellular concentration. Once ascertained Band 3 protein efficiency in anion exchange on SSc patients with or without ILD, MDA plasma levels, along with intracellular content of Reduced Glutathione (GSH) and Methemoglobin (MetHb), were measured to determine oxidative conditions, in SSc patients exhibiting ILD.

2. Results and Discussion

Figure 1A shows the time course for $\text{SO}_4^{=}$ uptake in control conditions (healthy patients) and in SSc patients with or without ILD. $\text{SO}_4^{=}$ transport in control erythrocytes increased steeply at the initial stage and reached equilibrium in 30 min, exhibiting a rate constant of $0.053 \pm 0.001 \text{ min}^{-1}$.

The rate constant for $\text{SO}_4^{=}$ uptake measured in erythrocytes from SSc patients with ($0.035 \pm 0.002 \text{ min}^{-1}$) or without ILD ($0.037 \pm 0.001 \text{ min}^{-1}$) was significantly lower than in control erythrocytes and $\text{SO}_4^{=}$ quantity trapped by the cells was significantly reduced as well. The reduction in both rate constant for $\text{SO}_4^{=}$ uptake and internalized $\text{SO}_4^{=}$ have been correlated to oxidative conditions detected in blood. Afterwards, only SSc-ILD patients were considered to complete the experimental plan. In this latter regard, in a separate experimental set, oxidative parameters have been measured and analyzed by one way ANOVA followed by Bonferroni's *post hoc* test: MetHb (figure 1B) levels in females and males with pulmonary fibrosis (SSc-ILD) were significantly different with respect to control, with levels in females significantly higher than in males. GSH levels (figure 1C) in both SSc-ILD female and male patients were significantly lower than control. MDA plasma levels (figure 1D), measured in both SSc-ILD female and male patients, were significantly higher than control, with MDA levels in males significantly higher than in females.

The present data show that erythrocytes from SSc patients exhibit a reduction in both anion exchange capability through Band 3 protein and in $\text{SO}_4^{=}$ content internalized by the cells during the observation time, with respect to erythrocytes from healthy patients. The extent of such decrease is comparable to that one recently observed in erythrocytes collected from healthy patients and then treated with hydrogen peroxide [19, 20]. In particular, the authors demonstrated that oxidative conditions, modeled by hydrogen peroxide within physiological range of concentrations (not higher than 300 μM), compromises anion exchange efficiency, though not inducing lipid peroxidation, thus demonstrating the sensitivity of the method (rate constant for $\text{SO}_4^{=}$ uptake measurement) to assess erythrocytes homeostasis. Hence, this method has been here used to monitor the impact of oxidative stress on SSc development.

The evidence that alterations in anion exchange capability through Band 3 protein may account for disease development has been recently provided [5]. The authors demonstrated that in erythrocytes collected from dogs with leishmaniasis, an oxidative stress-related disease, the rate constant for $\text{SO}_4^{=}$

uptake is significantly reduced with respect to control. Such evidence further corroborates the hypothesis that anion exchange through Band 3 protein measurement may account for development of oxidative stress-related pathologies and for homeostasis of the whole organism. In this respect, it is already known that Band 3 protein function is critically related

not only to anion balance, but also to oxygenation state of erythrocytes and, in turn, of the entire animal. In this scenario, Band 3 protein function assessment may also reasonably account for oxygenation state of SSc patients, namely those affected by Interstitial Lung Disease, a frequent complication of SSc development.

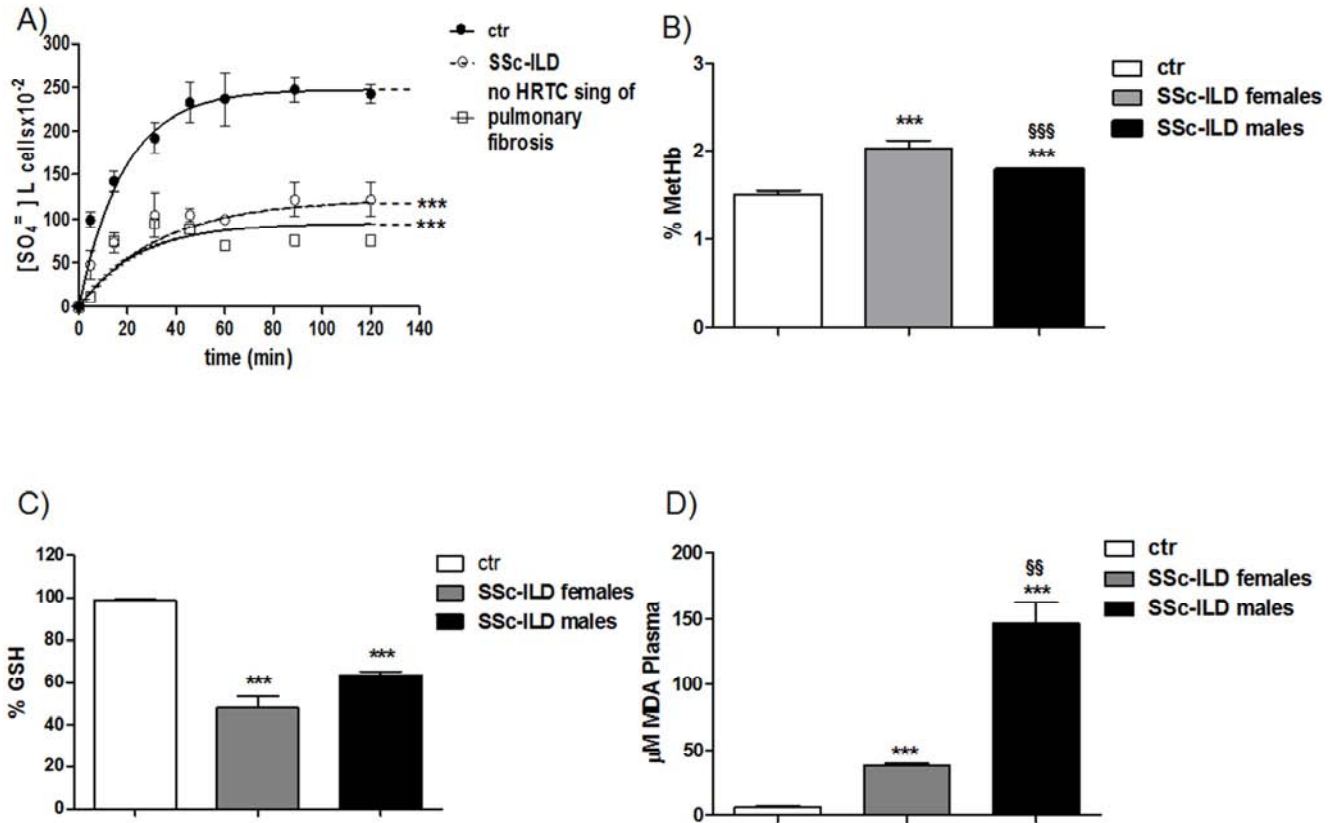


Figure 1. (A) Time course of SO_4^- uptake measured in erythrocytes from both healthy (control, $N=15$) and SSc patients ($N=6$ for female samples (F), $N=3$ for male samples (M)). Points represent the mean \pm SEM from separate experiments, where $***p<0.001$ significant different versus control. (B) MetHb levels in F and M patients with SSc-ILD were significantly different with respect to control ($***p<0.001$), with levels in F statistically higher than in M ($^{\$\$}p<0.001$). (C) GSH levels and MDA levels in both SSc-ILD F and M patients were significantly lower than control ($***p<0.001$). (D) MDA plasma levels measured in both F and M SSc-ILD patients. MDA levels in M were significantly higher than in F patients ($^{\$}p<0.001$).

Band 3 protein efficiency is reportedly known to account for erythrocytes deformability [12] an essential condition for blood flow. As vascular damage compromising microcirculation and leading, in turn, to ischemic episodes in SSc patients has been previously described, Band 3 protein function monitoring may be reasonably proposed as a potentially useful tool to verify erythrocytes behavior alterations during disease progression. Alterations at level of structural membrane properties, possibly revealed by Band 3 protein changes, may in fact lead to intravascular alterations occurring during SSc, contributing to blood hyperviscosity and exacerbating local ischemia [21]. More specifically, other authors [22] already reported structural alterations of Band 3 protein in SSc patients. In particular, reduced Band 3 protein expression levels correlating with phosphatidylserine externalization and, in turn, with eryptosis, has been ascertained.

The present report adds novel elements in this regard, as it has been recently demonstrated that progression of oxidative

stress-related pathologies, such as canine leishmaniasis [5], may not be associated with a reduction in Band 3 protein expression levels, but still associated to a reduced anion exchange capability through this membrane protein. Therefore, measurement of the rate constant for SO_4^- uptake may be considered as a more sensitive tool to monitor SSc development, and could be added to laboratory testing currently performed in clinical practice.

As a whole, the present findings may suggest a strong correlation between Band 3 protein efficiency in SSc patients and oxidative stress, supported by the evidence that levels of MetHb and MDA are significantly augmented in SSc patients, while GSH intracellular content is reduced. Band 3 protein in SSc patients, with or without ILD, is affected to the same extent, in spite of different MDA plasma levels, which confirms the sensitivity of the method. Furthermore, SSc development seems to be associated with destabilization of membrane protein arrangement, revealed by a reduced efficiency in anion exchange not dependent on

lipoperoxidation, which is in line with what already proven in human erythrocytes exposed to hydrogen peroxide [19].

With regard to rate constant for SO_4^- uptake determination (Figure 1A), sex differences have not been considered, due to samples availability. Nonetheless, as levels of MetHb and MDA (Figure 1B, D) were significantly different in male and female patients, a different response of erythrocytes to disease development according to sex differences should be hypothesized, thus contributing, in perspective, to gender medicine.

It is worthy of note that other authors have already correlated SSc development with oxidative stress by conducting their experiments on skin obtained from SSc patients [6]. The present report provides more knowledge about SSc development by monitoring erythrocytes homeostasis and proposing Band 3 protein anion exchange capability as a tool to fulfill the aim of, on one hand, verifying disease progression and, on the other hand, facilitating future investigations. Investigators have been hampered in the enrollment of patients to study skin fibrosis during SSc development [6]. Therefore, the use of the method proposed by the present report, being Band 3 anion exchange monitoring performed on aliquots of blood samples routinely withdrawn from patients, may serve as a biomarker or even a surrogate marker allowing easier study of patients with shorter disease duration.

One more novel aspect arising from the present report is disease duration. The study has been performed on patients with an average disease duration of 3.5 years, considered as the time from the first non-Raynaud symptom, which is shorter than what reported elsewhere [6]. In this light, the study of Band 3 protein efficiency in anion exchanging may be useful in defining a suitable time window for pharmacological treatment before skin lesions extension is exacerbated by disease progression.

In the present report phosphorylation pathways mediating Band 3 protein function [15, 16] have not been considered. As a matter of fact, as oxidative conditions associated with canine leishmaniasis have been already demonstrated to alter Band 3 protein anion exchange capability *via* endogenous antioxidant system and phosphorylative pathways [5], an involvement of this signaling pathways in SSc development can not be excluded and may be the subject of future studies.

3. Conclusions

The present report confirms that oxidant/antioxidant imbalance affects erythrocytes of SSc patients and shows that: i) the rate constant for SO_4^- uptake measurement is useful in monitoring erythrocytes homeostasis in oxidative stress-related pathologies such as SSc, namely at a duration of disease comprised between 3 and 4 years; ii) Band 3 protein could be considered as a candidate for drug development, currently involving antioxidants compounds; iii) erythrocytes oxidative state may be considered as a good biomarker for studying development of SSc and its complications.

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