Neutrophil-derived reactive oxygen species in SSc

Theresa C. Barnes¹, Marina E. Anderson¹, Steven W. Edwards² and Robert J. Moots¹

Abstract

Objective. Reactive oxygen species (ROS) are implicated in the pathogenesis of SSc. Neutrophils constitute a major source of ROS during inflammation. Here, we examined endogenous and stimulated ex vivo ROS production of SSc neutrophils compared with control neutrophils with and without prior priming with TNF-α.

Methods. ROS generation was measured using luminol-enhanced chemiluminescence. Neutrophils isolated from SSc patients and healthy controls were unprimed or were primed with TNF-α. ROS production was stimulated in vitro with phorbol 12-myristate 13-acetate (PMA) and formyl-met-leu-phe (fMLP). To examine the effects of serum mediators on ROS generation, control neutrophils were also stimulated with SSc or control serum.

Results. Neutrophil stimulation with PMA and fMLP resulted in a greater increase in ROS generation in SSc neutrophils compared with controls. However, unstimulated SSc neutrophils generated lower levels of ROS than controls. SSc neutrophils demonstrated an increased response to fMLP in the absence of in vitro TNF-α priming indicating priming of SSc neutrophils in vivo. SSc serum did not stimulate neutrophil ROS generation in vitro.

Conclusion. SSc neutrophils are primed for ROS generation. Neutrophils binding to activated endothelium in SSc, may induce local production of ROS, perpetuating endothelial dysfunction and mediating fibrosis.

Key words: systemic sclerosis, neutrophils, innate immunity, reactive oxygen species, oxidative stress.

Introduction

Reactive oxygen species (ROS) are produced by normal oxidative metabolism, but elevated levels can contribute to what is termed oxidative stress. Although many cell types can produce ROS, phagocytes are the predominant source of ROS during inflammation. There is accumulating evidence to suggest that oxidative stress may be important in the pathogenesis and perpetuation of SSc [1], a systemic CTD characterized by vascular dysfunction, immunological abnormalities and fibrosis. Endothelial dysfunction is thought to be pivotal in the pathogenesis of SSc [2].

ROS can damage proteins, lipids and DNA, leading to irreversible cell membrane damage and cell death via necrosis or apoptosis. Endothelial cells are particularly vulnerable to ROS-mediated damage, as they lack the protective enzyme catalase [3]. Exposure of endothelial cells to ROS results in damage to the cell membrane and degeneration and reduplication of the basement membrane, changes that are typically seen on histological samples from patients with SSc. ROS can also influence other processes that are implicated in the pathogenesis of SSc. For example, oxidative stress is reported to promote fibrosis and can lead to fragmentation of antigens, resulting in the exposure of neoantigens and the promotion of SSc-associated autoantigen responses [4].

The literature regarding neutrophil ROS generation in SSc is highly contradictory [5-8], perhaps in part reflecting the heterogeneity of the disease. However, it is more likely to reflect the different methodologies used to study this phenomenon. In this study, we used luminol-enhanced chemiluminescence to measure both intra- and extracellular ROS generation by SSc neutrophils following isolation using Polymorphprep resulting in minimal
neutrophil activation. Luminol-enhanced chemiluminescence is a sensitive assay that allows ROS generation to be monitored in real time. Our data show that SSc neutrophils are primed for ROS generation in response to the neutrophil stimulants formyl-met-leu-phe (fMLP) and phorbol 12-myristate 13-acetate (PMA).

**Methods**

The study was approved by the Sefton Local Research Ethics Committee, in accordance with the Helsinki Declaration. Informed written consent was obtained from patients with SSc [9] and from healthy volunteers. Thirty millilitres of heparinized venous blood was taken from the subjects.

Peripheral blood was separated into neutrophil and mononuclear cell fractions using Polymorphprep (as described in the manufacturer’s instructions). Contaminating erythrocytes were removed using ammonium chloride lysis buffer (KHCO₃ 3.4 mM, NH₄Cl 155 mM, EDTA 96.7 μM). Neutrophils were routinely examined for purity using morphological analysis of cytospins after staining with Rapid Romanowsky; purity was >95% immediately after isolation. Neutrophils were resuspended in Roswell Park Memorial Institute (RPMI) 1640 + 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) + 2 mM glutamine at a concentration of 5 × 10⁶ cells/ml.

**Luminol-enhanced chemiluminescence**

Two assays were used to measure chemiluminescence. For the microplate assay, neutrophils (1 × 10⁶/ml) from patients or controls were either primed with TNF (10 ng/ml) for 20 min or unprimed. Neutrophils (5 × 10⁴) were added to a 96-well, white plastic, non-coated plate. Luminol (disodium salt) was added to a final concentration of 67 μM, and PMA and fMLP were added to a final concentration of 100 ng/ml and 1 μM, respectively. Chemiluminescence was measured immediately on a PerkinElmer microplate reader every 30 s for 80 cycles. For the tube assay, 1 × 10⁶ neutrophils were diluted to 750 μl with Hank’s buffered salt solution (HBSS) warmed to 37 C. Luminol (sodium salt) dissolved in ddH₂O was added to a final concentration of 1 μM and 250 μl of either control or SSc serum was added as a stimulant. fMLP (1 μM, final concentration) was added to further samples as a positive control. Tubes were agitated and chemiluminescence was measured using a luminometer every 30 s for over 45 cycles. Data were normally distributed and therefore differences in means were analysed using the paired Student’s t-test.

**Results**

Clinical features of the patients are outlined in Table 1. One patient was taking MTX and two patients were taking MMF. Numbers were too small to allow subanalyses according to clinical phenotype.

**Table 1** Patient characteristics

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male : female)</td>
<td>1:12</td>
<td>1:12</td>
</tr>
<tr>
<td>Age, median (IQR), years</td>
<td>67 (58-70)</td>
<td>49 (44-54)</td>
</tr>
<tr>
<td>Disease subtype (lcSSc : dcSSc)</td>
<td>1:12</td>
<td></td>
</tr>
<tr>
<td>ANA positive (%)</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>ACA positive (%)</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Scl70 positive (%)</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>RNP positive (%)</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Renal involvement (%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Lung involvement (%)</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Pulmonary artery hypertension (PAH) (%)</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Modified Rodnan skin score (mRSS), median (IQR)</td>
<td>3 (2-5.75)</td>
<td></td>
</tr>
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Unstimulated neutrophil ROS generation is lower in patients with SSc compared with controls

Total unstimulated chemiluminescence, calculated as the area under the curve and corrected to healthy controls, was decreased on average by 25% in SSc neutrophils ($P = 0.0007, n = 13$) (Fig. 1).

PMA-stimulated neutrophil ROS generation is greater in patients with SSc compared with controls

In contrast to unstimulated rates of ROS, the total increase in chemiluminescence in response to PMA stimulation was greater in SSc neutrophils compared with healthy control neutrophils, both when unprimed ($P = 0.02$) and when TNF primed ($P = 0.01$). Healthy control neutrophils increased total chemiluminescence 4.9-fold (unprimed) and 5.0-fold (TNF primed) as compared with...
SSc neutrophils that increased 6.3-fold (unprimed) and 6.7-fold (TNF primed) (Fig. 2A).

fMLP-stimulated ROS generation is greater in unprimed SSc neutrophils compared with controls

Neutrophil responses to fMLP usually require priming with agents such as TNF and GM-CSF. However, the average increase in total chemiluminescence in response to fMLP in SSc neutrophils was 2-fold. This was significantly increased compared with healthy controls, which increased 1.7-fold on average (\(P = 0.046\)) (Fig. 2B). This suggests that neutrophils, isolated from SSc patients are primed in vivo. The response to fMLP of TNF-primed neutrophils from SSc patients was not significantly different from healthy controls.

Soluble factors in SSc serum do not affect neutrophil ROS generation

To determine whether serum factors were responsible for the decrease in ROS, healthy control neutrophils were exposed to SSc or healthy control serum and ROS generation was measured by chemiluminescence. No differences were found in the total chemiluminescence of either unprimed or TNF-primed neutrophils in response to SSc serum compared with healthy control serum in the absence of further stimulation (data not shown).

Discussion

The neutrophil stimulants fMLP and PMA resulted in the activation of higher levels of ROS in SSc neutrophils compared with control neutrophils. ROS generation in response to fMLP requires prior priming to increase the expression/affinity of fMLP receptors on the neutrophil surface. SSc neutrophils produced more ROS in response to fMLP when unprimed, suggesting that neutrophils are primed in vivo.

Neutrophil priming can occur in response to a number of different stimuli, including cytokines such as TNF-\(\alpha\), GM-CSF and IL-8. Interestingly, IL-8, which has been found in increased concentrations in SSc serum [10], is capable of priming ROS not only in response to fMLP, but also in response to PMA, unlike other priming agents [11]. Therefore IL-8 exposure in vivo may explain the increased response to both fMLP and PMA of SSc neutrophils. It is evident, however, that further priming in vitro can be achieved by the more powerful priming agent TNF-\(\alpha\). The fact that SSc neutrophils are primed for ROS generation suggests that these cells could contribute significantly to the oxidative stress that is present in SSc, promoting endothelial cell dysfunction, fibrosis and autoantibody production [1].

SSc neutrophils are hypofunctional in terms of spontaneous ROS generation in vitro. This may represent a primary functional deficit in SSc neutrophils or may reflect in vivo activation, and hence in vitro exhaustion. To explore this further, control neutrophils were exposed to SSc serum to determine whether this would stimulate ROS production. However, no such effect was observed. In addition, although unstimulated SSc neutrophils were hypofunctional in terms of endogenous ROS production, the fact that they demonstrated a greater increase in ROS in response to stimulants such as fMLP and PMA suggests that SSc neutrophils can be hyper-responsive in certain circumstances.

Our data are in agreement with another recently published paper that used an alternative and exclusively intracellular probe, DHR-123, to demonstrate a decrease in basal ROS production using a whole-blood assay with neutrophil ROS generation measured by FACS gated for
neutrophils only [5]. However, they did not find a significant difference between SSc and control neutrophil ROS production in response to PMA and did not examine the effect of fMLP, a more physiological neutrophil stimulator.

The literature contains several reports of increased ROS generation by SSc neutrophils in vitro in direct contradiction to our results [6–8]. The discrepancies may be explained by different methodologies that either used whole blood, and therefore were unable to discriminate the cellular source of ROS, or used neutrophil isolation techniques that may induce neutrophil activation. Our data indicate that SSc neutrophils have been primed in vivo, so that they would be more sensitive to perturbations that may occur during isolation procedures that inadvertently activate the cells. Previous data have shown that the method used in our study is minimally perturbing to the neutrophils [12].

In conclusion, SSc neutrophils have an abnormal functional phenotype ex vivo compared with control neutrophils. They are hypofunctional in terms of unstimulated ROS generation, but are hyperfunctional to fMLP- and PMA-induced ROS generation. The former observation may indicate exhaustion following in vivo activation and the latter may indicate in vivo priming. IL-8 could be a candidate priming agent. However, although neutrophils may be primed by soluble factors in SSc serum, SSc serum alone is not sufficient to activate neutrophils. Binding of primed neutrophils to leucocyte-binding proteins, which are increased on the surface of activated endothelium in SSc, however, could result in local production of ROS that would perpetuate endothelial activation and could lead to fibrosis.

**Rheumatology key message**
- SSc neutrophils are primed for ROS generation.

**Acknowledgements**

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**References**