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The emerging problem of oxidative stress and the role of anti-oxidants in systemic sclerosis

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Introduction

The pathogenesis of systemic sclerosis (SSc) is not fully understood, but vascular abnormalities are well recognised (1) and many of the clinical features of the disease are a result of ischaemic atrophy. Endothelial abnormalities occur early (2) and may drive the fibrotic disease process, but what initiates these abnormalities is not known. Modifications of the vascular system lead to an early dysfunction of the control of vascular tone (Raynaud's phenomenon) which then triggers a cascade of events in which there is increasing evidence to suggest that oxidative stress is a major player. Oxidative stress has been implicated in the pathogenesis of a large number of other disease states characterised by ischaemia (3), including inflammatory arthritis when increased pressures within the joint create a hypoxic environment (4). Oxidative stress is mediated by free radicals - atoms or molecules which contain one or more unpaired electrons. Free radicals are continually being produced in the body under physiological conditions, but under normal circumstances their effects are offset by antioxidant defences (5).

After the suggestion, in 1993, that free radicals are major contributors to the pathogenesis of SSc (6) a large number of papers have addressed the issue as to the role of free radicals in SSc. It seems highly plausible that free radicals contribute to vascular damage and jeopardise the function of the endothelial system, leading to immune system involvement and to fibroblast activation and eventually to tissue fibrosis.

This paper will consider the reasons why oxidant stress might be expected to contribute to tissue injury in SSc, the evidence that it does occur, and the experience to date with antioxidant therapy.

The reasons why oxidative stress *might* occur in SSc

Oxidative stress occurs when the body's normal balance between free radicals and antioxidants is disturbed, as a result of either excessive free radical formation, or antioxidant deficiency, or both (5). What is known about the pathogenesis and pathophysiology of SSc suggests two main factors as triggers of oxidative stress and of a multistep process leading to loss of function and injury to systems involved by SSc.

Ischaemic-reperfusion injury

Tissue injury is produced not only as a result of ischaemia, but also as a result of post-ischaemic reperfusion. Experiments in the feline intestinal model of ischaemic-reperfusion injury (7) implicated the free radical superoxide – superoxide dismutase (which scavenges superoxide) administered towards the end of the ischaemic period but before reperfusion attenuated the tissue damage. Figure 1 summarises

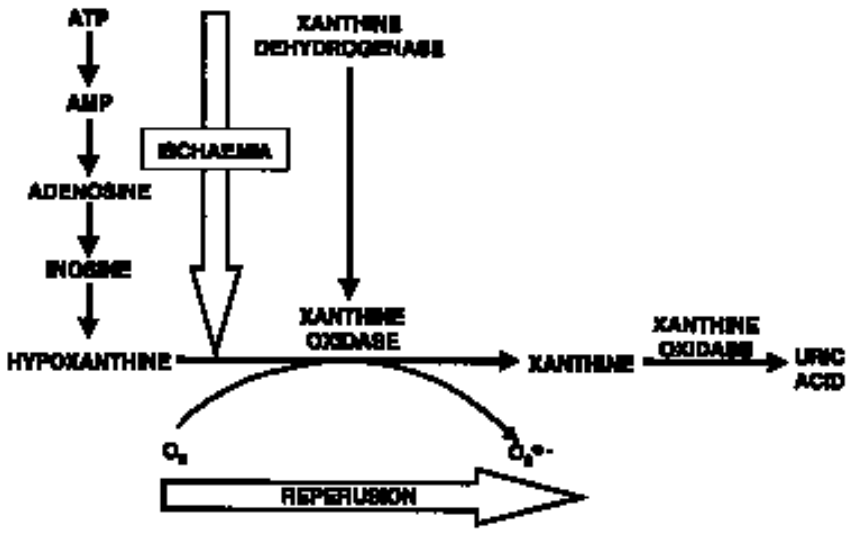

Fig. 1. Generation of superoxide (O_2^{Σ}) by ischaemia-reperfusion.

how superoxide is produced. During the ischaemic period hypoxanthine is produced from ATP, and the enzyme xanthine oxidase from xanthine dehydrogenase. When an influx of oxygen occurs with reperfusion, superoxide is produced. Recurrent episodes of ischaemia are a well recognised feature of SSc, most characteristically occurring in the digits as Raynaud's phenomenon. As disease progresses, structural vascular changes occur, and so the effects of an episode of vasospasm are superimposed upon a chronically compromised vasculature.

White blood cell activation. Xanthine oxidase, produced during ischaemia-reperfusion, may contribute to the upregulation of adhesion molecules (8) which is well described in SSc (9-11). These adhesion molecules can lead to entrapment of neutrophils, which when activated release oxidants as well as proteases. Thus, neutrophils can produce large quantities of reactive oxygen species (hydrogen peroxide [H_2O_2], superoxide, hydroxyl, peroxynitrite [$ONOO^-$]) that are injurious to the endothelium.

Endothelial activation/injury. Oxidative stress and its products perturb the function of the endothelial system, from a process of activation to injury and death. The upregulation of adhesion molecules is an early event facilitating lymphocyte trafficking through the endothelium and thus favouring their perivascular accumulation and interaction with fibroblasts. The direct effect of reactive oxygen species is also deleterious because these modify membrane lipids, proteins, carbohydrates, and DNA, causing cell injury and death.

An important role may be played by the free radical nitric oxide (NO). In certain situations, for example in the rheumatoid synovium, NO can be produced in excess via inducible NO synthase (iNOS) (12). It can then react with superoxide to form peroxynitrate and hydroxyl radicals, which are highly reactive and which will cause tissue injury. There is now increasing evidence to suggest that NO is produced in excess in SSc: increased NO release by peripheral mononuclear cells (13, 14), increased endothelial cell iNOS expression in sclerodermatous skin (15, 16) and elevated levels of serum nitrate/nitrite (11, 15) have all been reported. Therefore, the increase in NO production, that may be helpful in achieving vasodilation, paradoxically could be another contributor to oxidant stress in SSc (17).

In the last decade, there has been increasing interest in the capacity that endothelial cells possess to adapt to and to control oxidative stress. It has been demonstrated that endothelial cells adapt to the oxidative stimulation by a process that assures protection, and allows the inactivation of injurious factors. One hypothesis is that in SSc, endothelial cells are unable to 'react'to oxidant stress, either through a lack of antioxidant defence mechanisms or because these antioxidant mechanisms are exhausted (18).

Chromosomal breakage. A high rate of chromosomal breakages has been found in SSc (19). It has been suggested that reactive oxygen species have clastogenic activity and are able to induce chromosomal aberrations. The plasma of patients with SSc has been demonstrated to have increased levels of inosine triphophosphate and adenosine deaminase: these indicate high clastogenic activity and are biomarkers of oxidative stress as well (20). This suggests that oxidative stress may have a clastogenic activity on chromosomes and induce the chromosomal aberrations observed in SSc.

Autoantigen fragmentation. The increased production of reactive oxygen species during ischaemia-reperfusion may also cause novel autoantigen fragmentation, revealing immunocriptic epitopes in self antigens that could initiate the immune response with the generation of autoantibodies specific to the cleaved autoantigens (Topoisomerase I, NOR-90, U1-70kDa) (21). Thus free radicals might provide a link between the vascular and immune abnormalities of SSc (22).

Lipid peroxidation

The breakage of the physiological balance of the cellular membrane may lead to dysfunction of the affected system. The oxidation of low density lipoproteins (LDL) (23), the main constituents of the cellular membrane, is thought to be a key factor in the promotion of vascular disease, in particular atherosclerosis (24). Also, it has been shown that oxidative stress, through lipid peroxidation, is associated with structural and functional changes of the erythrocyte membrane in SSc (25). Bruckdorfer *et al*. reported that LDL isolated from patients with SSc demonstrated increased susceptibility to oxidation compared to LDL from healthy controls or from patients with primary Raynaud's phenomenon (26), raising the question as to whether an increased susceptibility to LDL oxidation might be a important determinant of vascular disease progression in SSc. Oxidised LDL is more immunogenic than native LDL and Simonini *et al.* and Herrick *et al*. have both reported increased concentrations of antibodies to oxidised-LDL in patients with SSc (27, 28), further implicating oxidant stress in the pathogenesis of SSc via oxidation of LDL. Simonini *et al.* also reported an increased concentration of antibodies against oxidised LDL in the earlier phases of either the diffuse or the limited SSc subset (29).

Toxins

SSc or a sclerodermatous disorder is precipitated in some patients by exposure to environmental toxins, for example vinyl chloride, silica and bleomycin. We do not fully understand how the toxin triggers the disease process, but it is well recognised that a number of environmental chemicals (xenobiotics) exert their effects via oxidant stress, either by free radical generation or antioxidant depletion, and that these include bleomycin (30). Interestingly it has been suggested that anilide-induced oxidative stress contributed to tissue injury in patients with toxic oil syndrome, a syndrome with many similarities to SSc (31). It therefore seems possible that toxin-induced oxidant stress occurs in some patients with SSc. Thus several mechanisms could account for oxidant stress in SSc (Fig. 2). Once produced, free radicals could cause different types of tissue injury but the two which have attracted the

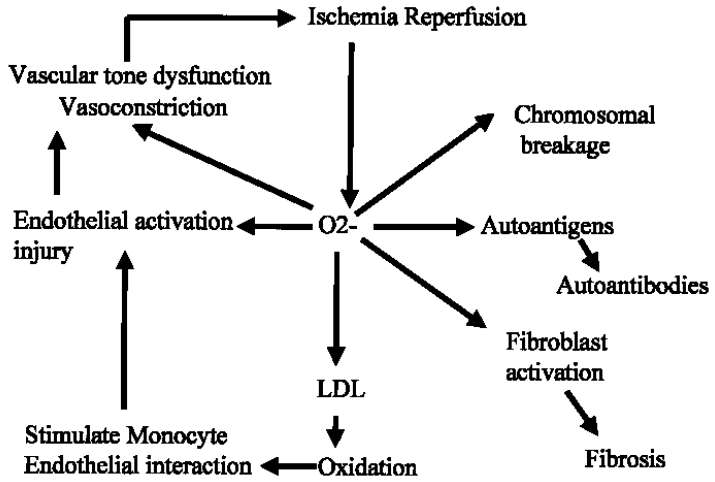

Fig. 2. The possible mechanisms of tissue injury in SSc by oxidative stress, and the role of superoxide(O_2^{Σ}).

most interest in SSc are (a) injury to endothelial cells though lipid peroxidation of cell membranes (causing vascular damage) and (b) fragmentation of nuclear antigens (causing immune dysfunction), as already referred to above (21). But what is the evidence?

The evidence that oxidant stress *occurs* in SSc

To identify oxidant stress, we need to look for evidence of excessive free-radical production and/or evidence of antioxidant deficiency. One problem is that, because most free radicals are very short-lived, it is difficult to measure them directly, and so what is looked for is indirect evidence of free radical mediated injury. Different investigators have used different methodologies to tackle this issue in SSc. Most studies to date have been cross-sectional, comparing patients with SSc to healthy control subjects and sometimes also to patients with primary Raynaud's phenomenon (PRP), in whom episodes of Raynaud's are generally considered to be entirely reversible, not leading to tissue injury.

Lau *et al*. studied oxidant stress in SSc in relationship to white blood cell activation. Plasma thiol concentrations were reduced in patients with SSc (and also in those with vibration white finger) compared to controls, suggestive of increased free radical production, and these reduced thiol levels were found in association with white blood cell activation (32). The same group of investigators then reported raised levels of malondialdehye (MDA), which is a marker of lipid peroxidation, in patients with SSc (and also in those with PRP): again this was related to increased polymorphonuclear cell activity (33). Therefore both of these studies suggested polymorph activation to be a source of free radicals in SSc.

Blann *et al.* also reported increased lipid peroxidation in SSc (34). Although Herrick *et al*. did not confirm increased lipid peroxidation, lipid isomerization as measured by the molar ratio of the 9-cis,11-trans isomer of linoleic acid to the parent fattty acid (another marker of free radical associated activity) was increased in patients with SSc (35). This increased activity of the lipid isomerization pathway was found in association with reduced circulating concentrations of the micronutrient antioxidants ascorbic acid and selenium, and a hypothesis resulting from this study was that patients with SSc were predisposed to oxidative stress because of impaired antioxidant defences (35). The same group of investigators could not demonstrate evidence of reperfusion injury after a thermal stress test in patients with SSc, although there were a variety of possible reasons for this including the insensitivity of the free radical markers used, and the fact that blood was sampled after a relatively mild cold stress to the hand from the antecubital fossa (36).

The finding of reduced concentrations of circulating micronutrient antioxidants in patients with SSc was confirmed by Lundberg *et al*.; in this study concentrations of vitamin E and beta-carotene were reduced in addition to those of vitamin C and selenium (37). The reason for the reduced micronutrient antioxidant levels is not known. It is not thought to be due to poor dietary intake (38), nor to malabsorption in the case of the highly water soluble vitamin C (39).

Stein *et al.* pointed out that it is difficult to measure lipid peroxidation *in vivo* (40). These investigators therefore used another approach to look for oxidative stress and reported increased levels in patients with SSc of a marker of free-radical catalysed peroxidation of arachidonic acid – a urinary metabolite of F_2-isoprostanes (40). Concentrations of an F_2-isoprostane were also found to be raised in bronchoalveolar lavage fluid from patients with SSc-associated fibosing alveolitis, and also from patients with cryptogenic fibrosing alveolitis, compared to control subjects, suggesting that oxidative stress is likely occur in SSc associated lung disease (41).

Other investigators have examined superoxide. Morita *et al*. reported increased activity of plasma superoxide dismutase (42) and Sambo *et al*. in an *in vitro* study found that monocytes cultured from patients with SSc produced increased amounts of superoxide (43). Both groups of investigators interpreted their findings as further support for oxidative stress in SSc.

As already stated, very recently Solans *et al*. in a study including patients with SSc, PRP and healthy control subjects reported that lipid peroxidation was increased in the SSc cohort and that this was associated with reduced erythrocyte membrane fluidity (25). This study therefore provided evidence of another mechanism whereby free-radical mediated injury might jeopardise microvascular flow.

Perhaps the most direct evidence that free radicals actually cause tissue injury in SSc was provided by Cotton *et al.* (16). In a study of forearm skin biopsies from patients with different grades of disease, an increase in forearm skin grade was associated with

increased expression of iNOS, and this increased iNOS expression was paralleled by an increase in endothelial expression of nitrotyrosine, a marker of NO-mediated free radical damage (16). Although few of these studies are directly comparable, all are in different ways supportive of the hypothesis that oxidative stress contributes to tissue injury in SSc and that this is likely to be causing vascular and internal organ damage, as well as changes to the cellular components of the blood.

Antioxidants in SSc

Despite the above evidence supportive of oxidant stress in SSc, very little work has been undertaken to examine the efficacy of antioxidant therapy.

Niwa *et al*. reported that liposomal encapsulated superoxide dismutase benefitted 3 patients with SSc (44). This anecdotal observation has been followed by 2 controlled clinical trials of antioxidant therapy. Denton *et al*. conducted a parallel group study of 40 patients with Raynaud's phenomenon (20 of whom had SSc), randomised to receive either the antioxidant probucol 500 mg daily or nifedipine 20 mg daily. Treatment with probucol resulted in a reduction in the frequency and severity of Raynaud's attacks, and a rise in the oxidation lag time, reflecting a protective effect on LDL (45).

A recently reported study of a combination of micronutrient antioxidants and allopurinol (the latter given to block superoxide via xanthine oxidase) in 33 patients with limited cutaneous SSc showed no benefit from active treatment. While this may mean that antioxidant therapy is ineffective, one conclusion from the study was that to be effective, antioxidant therapy probably needs to be given early in disease, before irreversible tissue injury has occurred (46).

Antioxidant therapy is safe and in the absence of other effective treatment options deserves further study in patients with SSc. However, studies aimed at modifying vascular disease progression in SSc are difficult to mount, primarily due to the difficulty in measuring outcome as most of the measures currently used (number of digital ulcers, patient diaries) are subjective and/or insensitive to change.

Conclusion

There is now a substantial body of evidence to suggest that oxidant stress occurs in SSc, possibly causing vascular injury and predisposing to autoimmunity. While there is so far very little evidence that antioxidants are effective therapy, this is in part due to the difficulties in mounting clinical trials. Future trials should include patients with early disease, and measures of outcome which are sufficiently robust and sensitive to detect small but clinically important changes over time.

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