Abnormal nitric oxide metabolism in systemic sclerosis: increased levels of nitrated proteins and asymmetric dimethylarginine

A. Dooley, B. Gao, N. Bradley, D. J. Abraham, C. M. Black, M. Jacobs and K. R. Bruckdorfer

Objectives. Endothelial dysfunction is a primary event in systemic sclerosis; however, the aetiology of events and the role of nitric oxide (NO) is still unclear. The aim of the present study is to investigate whether there are abnormalities in NO metabolism in plasma from patients with primary Raynaud's phenomenon (RP) and in the pathogenesis of systemic sclerosis (SSc): limited SSc (ISSc) and diffuse (dSSc). We also wanted to investigate the effect of factors within patients' SSc serum on NO metabolism in human microvascular endothelial cells (HMECs).

Methods. Plasma (n = 89) or serum (n = 80) was assayed for total nitrate and nitrite (NOx), nitration of proteins and the NO inhibitor asymmetric dimethylarginine (ADMA). HMECs were treated with patients' SSc serum and assayed for indicators of NO metabolism.

Results. Plasma NOx was elevated in patients with RP or ISSc (P < 0.002), but not in patients with dSSc, compared with controls. Nitrated proteins in plasma, however, were found to be very high in dSSc patients (P < 0.03), compared with RP, ISSc or controls. Patients with dSSc also showed increased levels of serum ADMA (P < 0.05). The high level of nitrated proteins in dSSc was strongly associated with the severity and duration of dSSc disease. Skin biopsy sections from dSSc patients also showed enhanced nitrotyrosine staining compared with controls. In HMECs, pre-incubation with SSc serum impaired the activity of nitric oxide synthase (NOS) but not the expression of inducible or endothelial NOS. SSc serum also induced a reduction in intracellular cGMP synthesis, and NOx production in the cell culture medium, but was not associated with increased cell cytotoxicity.

Conclusions. NO formation is increased in patients with primary RP or ISSc, but nitration of proteins and elevated ADMA is a particular feature of dSSc and may reflect abnormal NO regulation and/or contribute to endothelial dysfunction in SSc.

Key words: Systemic sclerosis, Primary Raynaud’s, Nitrate, Nitrated proteins, ADMA, Endothelial dysfunction.

Systemic sclerosis (SSc) is a multisystem autoimmune disease characterized by an excessive deposition of extracellular matrix both in the skin and the internal organs. There are several classified clinical subgroups, including limited (ISSc) and diffuse (dSSc) cutaneous SSc, which reflect the nature of the disease in their degree of skin sclerosis, immunological profile and microvascular dysfunction [1, 2]. Endothelial activation and damage are primary events throughout the course of the disease, and prominently feature in the related Raynaud’s phenomenon (RP), which is distinguished by cold-induced digital ischaemic attacks and vasospasms [3, 4]. The nature of the factors that induce endothelial dysfunction are still unclear; however, there are several serological biomarkers that reflect the vasculopathy of the disease. These include the vasoconstrictor endothelin [5], cell adhesion molecules such as selectin [6], anti-endothelial antibodies [7] and controversially the vasodilator nitric oxide [8–12].

The free radical nitric oxide (NO) is a potent vasodilator and is synthesized from L-arginine by NO synthase (NOS). Three main isoforms of NOS have been identified with a constitutive expression in neuronal (nNOS or NOS 1), endothelial (eNOS or NOS 3) and several other cell types. Furthermore, an inducible expression (iNOS or NOS 2) in response to a variety of stimuli is possible, with NO-mediated signalling apparent in the skin [13]. NO, however, is considered to have a biphasic effect in physiological and pathological conditions, being both beneficial and detrimental depending on the concentration and local environment [14, 15]. Regulation of NO by endogenous levels of the NOS inhibitor asymmetric dimethylarginine (ADMA) has also recently been proposed [16]. In most biological situations NO is largely oxidized to nitrate (NO3⁻) and nitrite (NO2⁻), with the measurement of total nitrate and nitrite (NOx) production, as well as ADMA levels, seen as a reflection of endothelial dysfunction in many diseases [16, 17]. Studies involving NOx production in SSc, however, have shown conflicting results [8–12], while recently elevated ADMA levels have been demonstrated in RP [18].

Another indirect parameter of abnormal endothelial function has been the measurement of nitrated proteins caused by the reaction of NO with the free radical superoxide anion to form peroxynitrite (ONOO⁻). The latter is a potent oxidizing reagent, which nitrates tyrosine residues of proteins, changing the structure and functions of normal proteins and causing tissue damage [19]. These modifications may play an important role in the pathology...
of inflammatory diseases [20, 21], and indeed evidence of enhanced staining of nitrated proteins in SSc skin has been reported [11] although the circulating level of these proteins is unknown. The suggestion that free radicals and oxidative stress may be involved in the pathogenesis of these diseases is supported by studies showing increased levels of superoxide anions [22, 23], antibodies against oxidized low-density lipoproteins [24, 25] and increased urinary F2-isoprostanes [26] from patients with SSc. Indeed, we have demonstrated in our earlier work low levels of plasma antioxidants in scleroderma, and the beneficial effects of the potent antioxidant probucol in RP [27, 28]. Subsequently, antioxidant therapy has been proposed as a possible treatment in SSc [29].

The aim of our present study was to investigate and clarify whether circulating factors of NO metabolism (nitrate and nitrite, protein-bound nitrotyrosine and ADMA) reflect the clinical features of RP and systemic sclerosis disease. We also investigated whether, conversely, factors present within the serum of SSc patients could affect human dermal microvascular endothelial NO metabolism.

Materials and methods

Study subjects

Patients. RP was defined by triphase colour changes (pallor-cyanosis-suffusion) affecting the extremities, precipitated by cold or emotion. Patients with primary RP were enrolled into the study, which was approved by the Royal Free Hospital Ethical Practices Subcommittee, and were further defined as having a negative autoantibody profile with no clinical evidence of a connective tissue disease. Some of the patients were also associated with SSc. These patients fulfilled the American Collage of Rheumatology (formerly, the American Rheumatism Association) preliminary diagnostic criteria for SSc [30], comprising patients with dSSc and with ISSc. Information regarding clinical features, such as skin score, onset of disease, sex and age are summarized in Table 1. Skin score was assessed according to the modified Rodnan method [31]. Four patients with RP, ten with ISSc and seven with dSSc were treated with vasodilators (calcium channel blockers, angiotensin-converting enzyme inhibitors and/or prostanoids). Five patients with ISSc and six patients with dSSc required further treatment with low-dose corticosteroids and immunosuppressive therapy.

One patient in the RP group, one in the ISSc, one in the dSSc and one of the controls were current smokers. All subjects provided informed consent to participate in the study.

Plasma and serum. Plasma and serum samples were obtained from patients or healthy individuals using vials containing EDTA or clot activator and stored at –80°C. Plasma measurement of endogenously generated nitrite, and nitrate in particular, is complicated by several factors. The most significant is the contribution to nitrite and nitrate in the plasma pool from the diet, for example from leafy green vegetables, preserved meats and drinking water. In order to minimize the effect of the diet, subjects for nitrate and nitrite plasma measurement (and also nitrated plasma proteins) were maintained on a low-nitrate, low-nitrite diet, such as bread, cereals, pasta, cheese, fruit drink and milk for three full days, and then fasted overnight, prior to collection of blood.

Metabolic studies

Chemicals. All chemicals unless otherwise indicated were purchased from Sigma (UK), or Invitrogen (UK).

Measurement of total nitrate and nitrite (NOx), and nitrated plasma proteins. Concentrations of total nitrate and nitrite (NOx) and nitrite (NO2) were measured in plasma or endothelial conditioned media by a NO chemiluminescence detector (NOA 280, Sievers). Plasma samples (1:10 dilution) were filtered through ultrafare-4 filters and centrifuged for 1 h at 2500 g. After removing high-molecular proteins, plasma samples, together with nitrate (NO3) standard solutions, were incubated with Tris buffer (20 mM pH 7.6) containing nitrate reductase 10 nM, NADPH 40 μM and flavine adenine dinucleotide (FAD) 1 μM, for 1 h at 37°C to convert total NO3 into NO2. Samples were then reduced to NO in a purge vessel containing potassium iodide (1%) in acetic acid. Levels of NO2 in plasma were also measured as described without previous reduction with nitrate reductase. Levels of NOx in endothelial conditioned medium were measured after the cells were pre-incubated with control or SSc serum (20% in MCDB 131 medium) for 24 h. Cells were washed twice with phosphate-buffered saline (PBS), and further grown for 6 or 24 h in MCDB 131 medium.

Table 1. Comparison of NO metabolism in plasma or serum from patients with different subsets

<table>
<thead>
<tr>
<th></th>
<th>CON</th>
<th>PRP</th>
<th>ISSc</th>
<th>dSSc</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>25</td>
<td>19</td>
<td>24</td>
<td>21</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>48±5</td>
<td>45±4</td>
<td>51±3</td>
<td>52±4</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>19/6</td>
<td>14/5</td>
<td>18/6</td>
<td>15/6</td>
</tr>
<tr>
<td>Duration of disease (yr)</td>
<td></td>
<td>9.6±2.5</td>
<td>12.8±1.7</td>
<td>9.5±1.4</td>
</tr>
<tr>
<td>Skin score</td>
<td>–</td>
<td>9.6±2.5</td>
<td>12.8±1.7</td>
<td>9.5±1.4</td>
</tr>
<tr>
<td>NOx (μM)</td>
<td>23.4±1.6</td>
<td>40.6±2.6*</td>
<td>44.9±5.3*</td>
<td>27.4±1.8</td>
</tr>
<tr>
<td>NO2 (μM)</td>
<td>0.31±0.05</td>
<td>0.38±0.05*</td>
<td>0.31±0.04</td>
<td>0.31±0.05</td>
</tr>
<tr>
<td>Nitrated proteins (ng/mg total plasma proteins)</td>
<td>5.8±1.0</td>
<td>7.0±1.0</td>
<td>8.1±1.4</td>
<td>48.7±14.1*</td>
</tr>
<tr>
<td>Plasma total proteins (mg/ml)</td>
<td>680±17</td>
<td>638±14</td>
<td>662±20</td>
<td>632±18</td>
</tr>
<tr>
<td><strong>Serum</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number</td>
<td>19</td>
<td>18</td>
<td>22</td>
<td>21</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>43±3</td>
<td>44±5</td>
<td>57±2</td>
<td>54±3</td>
</tr>
<tr>
<td>Sex (F/M)</td>
<td>10/9</td>
<td>13/5</td>
<td>15/7</td>
<td>15/6</td>
</tr>
<tr>
<td>Duration of disease (yr)</td>
<td></td>
<td>5.9±2.6</td>
<td>9.3±2.0</td>
<td>7.8±1.4</td>
</tr>
<tr>
<td>Skin score</td>
<td>–</td>
<td>9.7±1.4</td>
<td>9.7±1.4</td>
<td>14.2±2.0</td>
</tr>
<tr>
<td>ADMA (μmol/l)</td>
<td>0.67±0.04</td>
<td>0.67±0.04</td>
<td>0.78±0.04</td>
<td>1.00±0.14*</td>
</tr>
</tbody>
</table>

CON, healthy normal controls; RP, patients with primary Raynaud’s phenomenon; ISSc, patients with limited cutaneous SSc; dSSc, patients with diffuse cutaneous SSc. The results are expressed as the mean±s.e.m.

*P<0.05 was considered statistically significant.
Determination of nitrated plasma proteins was measured by competitive enzyme-linked immunosorbent assay (ELISA) that had been previously developed in this laboratory [32]. Nitrated bovine serum albumin (nitro-BSA), used as a standard nitrated protein, was prepared by three additions of an alkaline stock solution of peroxynitrite to a final concentration of 1 mM. The concentration of 3-nitrotyrosine in nitro-BSA was determined by absorbance (438 nm at pH 9.0) using a molar extinction coefficient of 4300 M$^{-1}$ cm$^{-1}$ and was in the range of 3–6 mol nitrotyrosine/mol protein.

**Measurement of serum ADMA.** Concentrations of ADMA were measured in a separate group of human serum samples by using a previously unavailable commercial competitive ELISA [33], according to the protocol provided by the manufacturer (DLD Diagnostika, Germany). In brief, 96-well microtiter plates were coated with ADMA bound to albumin. ADMA standards, blanks, samples and positive controls were pre-acylated before adding to the wells, followed by rabbit anti-N-acetyl-ADMA and incubated for 15–20 h at 2–8 °C. After incubation, the wells were washed and subsequently the secondary antibody, anti-rabbit IgG conjugated to peroxidase, was added followed by a 1 h incubation at room temperature. After washing, colour development was initiated by addition of the tetramethylbenzidine substrate solution. After 20–30 min the reaction was stopped by addition of 0.3M sulphuric acid. The absorbance was read at 450 nm by an automated microplate reader (Dynex Technologies, UK) and the standard curve used to determine the ADMA concentration. The ADMA concentrations obtained and the performance of the ELISA have been found to be consistent with other widely applied methods used to quantify ADMA, such as gas chromatography-mass spectrometry and liquid chromatography-mass spectrometry [33].

**Nitrated protein expression.** Punch skin biopsies (4 mm) were taken from the clinically affected skin of the forearm of two healthy control individuals matched for age and sex. In brief, skin sections were used to investigate for the presence of nitrated proteins by immunohistochemistry using a polyclonal anti-nitrotyrosine antibody (1:1000; TCS Biologicals, UK). After incubation with a secondary biotinylated goat anti-rabbit anti-nitrotyrosine antibody (1:1000; TCS Biologicals, UK). After incubation with a secondary biotinylated goat anti-rabbit antibody (Dako, UK), and then the streptavidin ABC-horseradish peroxidase system (Dako, UK), sections were visualized using Vector AEC peroxidase substrate and counterstained with haematoxylin (Vector Labs, UK) according to the instructions of the manufacturer.

**Cell culture studies**

**Cell culture.** Cells from the human dermal microvascular endothelial line (HMEC) were cultured in MCDB 131 medium, supplemented with 20% fetal calf serum (FCS), 4 mM l-glutamine, 20 μg/ml endothelial cell growth supplement and penicillin/streptomycin. Cells were maintained in a humidified atmosphere of 5% CO$_2$ at 37°C. Cells were grown in 96, 24, 12, and 6-well plates for up to 24 h with control or SSc serum (20% in MCDB 131 medium). HMECs were generously donated by Professor F. Candal (Centers for Disease Control and Prevention, GA, USA).

The human dermal fibroblast cell line AG1518 (FIBROBLASTS) was also used in the current study. This cell line was established from fetal foreskin (Coriell Cell Repositories, NJ, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS, 2 mM l-glutamine and penicillin/streptomycin.

**Cell survival assay.** After serum incubation, cells were washed with PBS and MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) was added at a final concentration of 0.5 mg/ml medium for 80 min according to a modified method by Mosmann [34]. Briefly, MTT precipitate produced by viable cells was dissolved in dimethyl sulphoxide (DMSO) and measured at a wavelength of 550 nm. Survival is defined in terms of optical density units.

**NOS enzyme activity assay.** Serum treated cells were homogenized in ice-cold lysis buffer [25 mM Tris-HCl pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethyleneglycoltetraacetic acid (EGTA), underwent a freeze-thaw cycle, and then were spun for 5 min at 10 000 g and the lysate removed. Total NOS activity was determined by the conversion of [14C]-L-arginine (Amersham Biosciences, UK) to [14C]-L-citrulline using a commercially available NOS assay activity kit (Calbiochem, UK). In brief, lysates were incubated in the presence or absence of l-NAME (N$^\text{N}$-nitro-L-arginine-methyl-ester; 100 μM) with a reaction buffer [25 mM Tris-HCl pH 7.4, 3 μM tetrahydrobiopterin, 1 μM FAD, 1 μM flavin mononucleotide (FMN), 1 mM NADPH, 10 μM [14C]-arginine, 3 mM CaCl$_2$ and water] and incubated at 37°C for 60 min. The reaction was then stopped by the addition of ice-cold HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 50 mM, pH 5.5), EDTA (5 mM) and equilibrated resin to each sample. [14C]-L-citrulline was eluted from the samples and radioactivity quantified in a liquid scintillation counter. Protein concentrations of cell lysates were determined by the BCA protein assay (Pierce, UK), and retrieved [14C]-l-citrulline values were normalized to total protein.

**Measurement of intracellular cyclic guanosine monophosphate (cGMP).** After serum incubation, cells were washed with PBS and stimulated in the presence or absence of 2-(N,N-diethyleniamino)-diazeneolate-2-oxide (DEANO; Alexis Biochemicals, UK; 10 μM) after 20 min of pre-incubation with a Hanks Balanced Salt Solution (HBSS) buffer containing 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM), L-arginine (100 μM), and superoxide dismutase (SOD; 150 U/ml). The media was removed and cells frozen in ice-cold trichloroacetic acid solution (TCA; 7.5% in PBS) at −20 °C. The TCA extract was collected from thawed samples, and the cell remnant removed from the wells by adding sodium dodecyl sulphate (SDS; 0.1% in 0.1 M NaOH). The TCA extract was further analysed for cGMP by using a commercial competitive ELISA (Cayman Chemicals, MI, USA), while NaOH-solubilized samples were used for protein determination by the BCA protein assay (Pierce, UK).

**Western blotting for NOS protein expression.** Cells were washed with PBS and homogenized in lysis buffer [1 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton, 1 mM phenylmethylsulphonylfluoride (PMSF), 1 mM NaF, 1 mM Na orthovanadate, 1 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin]. The samples were centrifuged at 10 000 g for 5 min and the supernatant was separated from the pellet. Protein concentrations of the supernatant samples were determined using the BCA protein assay (Pierce, UK). For all gels, 20 μg of sample protein were run on a NuPAGE 4–12% gradient gel under reducing conditions. Proteins were transferred onto nitrocellulose membranes and incubated in a blocking buffer (5% non-fat dried milk, 0.1% Tween 20) for 1 h. Briefly, the membranes were incubated overnight with an anti-eNOS monoclonal antibody (1:500; BD Biosciences, UK), anti-iNOS monoclonal antibody (1:500; BD Biosciences, UK), or additionally anti-β-actin polyclonal (1:500; Sigma, UK). The membranes were probed with the appropriate horseradish peroxidase-conjugated secondary antibody with detection by West Dura chemiluminescent substrates (Pierce, UK) and visualized using the Alpha Innotech imaging system.
Extraction of RNA and reverse transcriptase polymerase chain reaction (RT-PCR). Cells were washed with PBS and homogenized in RLT lysis buffer (Qiagen Ltd, UK). The QIAamp RNA (Qiagen Ltd, UK) mini protocol for isolation of total RNA from cultured cells was used according to the manufacturer’s instructions. The concentration and purity of RNA was determined so that 1 μg of RNA could be used to start RT-PCR.

RT-PCR was performed using the one-step RT-PCR kit (Qiagen Ltd, UK). Primers specific for human iNOS 237 bp (TCT TGG TCA AAG CTG TG- (forward) and CAT TGC CAA ACG TAC TGG TC- (reverse)), human eNOS 438 bp (GAA GAG GAA GGA GTC CAG TAA CAC AGA C- (forward) and GGA CTT GCT GCT TTG CAG GTT TTC- (reverse)) and human β-actin 436 bp (CCA GAG CAA GAG AGG CAT CC- (forward) and CTG TGG TGG TGA ACG TGT AG- (reverse)) were used. The thermal cycling conditions were as follows: 1× (50°C, 30 min), 1× (95°C, 15 min), then n cycles of [(94°C, 1 min), (x°C, 1 min) and (72°C, 1 min)], followed by 1× (72°C, 10 min). The annealing temperature (x) for the primers were 60, 60 and 60°C, respectively. The number of cycles (n) for each set of primers was 35, 35 and 30, respectively. PCR products were resolved by agarose gel (1%) electrophoresis and stained with ethidium bromide. The products were then visualized under UV light using a camera imager (Alpha Innotech, CA, USA) and arbitrary integrated density values (IDV) recorded.

Data analysis. Data were expressed as mean±standard error of the mean (S.E.M). Comparisons of multiple means were made by using one-way ANOVA followed by the Student’s Newman–Keuls test. Correlation and regression were performed using the ‘least squares’ method in the Microsoft Excel program. P<0.05 was considered statistically significant.

Results

Plasma NOx and NO2 levels

The levels of NOx, and also NO2, were assessed in the subgroups of patients with primary RP, ISSc, dSSc and healthy normal volunteers: the mean values of each group are shown in Table 1. A significant increase in NOx was found in plasma from patients either with primary RP or ISSc (P<0.002), compared with normal controls (Table 1, Fig. 1a). However, the level of NOx in the dSSc group was similar to that of controls. No differences were observed among the patient plasma groups (Table 1, Fig. 1a).

Nitration of proteins and ADMA concentrations

There was no difference in total protein among the patient plasma groups (Table 1). In the healthy controls, nitration of proteins (protein-bound 3-nitrotyrosine) were found at the level of 5.8±1.0 ng/mg. Similar levels of nitrated proteins was noted in the patient groups of primary RP and ISSc, but a significantly elevated amount was demonstrated in the dSSc group (48±14 ng/mg, P<0.03) (Table 1, Fig. 1b). The levels of nitration of proteins in dSSc patients were found to be strongly associated with skin score (P<0.002), a marker of the severity of the disease (Table 1, Fig. 2a); patients with high skin score show high levels of 3-nitrotyrosine in their plasma. The regression equation of 3-nitrotyrosine level (Y) on skin score (X) is, Y = 4.77X – 48.9, and its multi-R² is 0.769. There is also a strong association with the duration of onset of dSSc disease (Table 1, Fig. 2b). Much higher levels of 3-nitrotyrosine were found at the earlier stage (less than 7 yr duration, 102.6±23.8 ng/mg, P<0.005) of the disease. In the patients where the duration of the disease was at a later stage (over 7 yr duration, 8.4±2.3 ng/mg); however, the level of nitrated proteins was found to be within the normal range (Fig. 2c).

Patients with dSSc also featured significantly increased ADMA levels (1.0±0.14 μmol/l vs controls 0.67±0.04 μmol/l; P<0.05). Levels of ADMA were similar to controls in the patient groups of primary RP and ISSc (0.67±0.04 μmol/l and 0.78±0.04 μmol/l, respectively) (Table 1, Fig. 1c).

Skin biopsy sections stained for nitrotyrosine showed there was little evidence of nitrated proteins in control skin (Fig. 3a), but enhanced nitrotyrosine expression was seen particularly in the epidermis of dSSc skin (Fig. 3b), around microvessels in the dermis (Fig. 3c) and associated with fibrous material (Fig. 3d).
NOS activity and NO production in cultured endothelial cells

Since there were abnormalities in nitric oxide metabolism in plasma from patients with primary RP, ISSc and dSSc, we addressed the hypothesis that factors in the patients’ sera could affect endothelial function. We investigated the effect of serum from SSc patients on nitric oxide metabolism in cultured human endothelial cells. Treatment of HMECs with SSc serum for 24 h reduced NOS activity in cell lysates compared with control serum (Fig. 4a). The synthetic NOS inhibitor L-NAME also decreased NOS activity both in cells incubated with control serum and to a greater extent with SSc serum (Fig. 4a).

The reduction in endothelial NOS activity after incubation with SSc serum was associated with a significant decrease in intracellular cGMP levels (a reflection of NO bioactivity) and was not restricted to cell type (Fig. 4b). Baseline cGMP levels in both cell types were increased after stimulation with DEANO or bradykinin; however, there was no effect of SSc serum on stimulated values (data not shown).

Accordingly, we determined the level of NOx metabolites in the endothelial culture medium 6 and 24 h after pre-supplementation with SSc serum. NOx production was significantly reduced in SSc pre-treated samples in comparison with the controls at both 6 and also 24 h (Fig. 4c).

Endothelial NOS protein and gene expression

Protein and gene expression were assessed in HMECs incubated with control or SSc serum. Western analysis revealed that the expression of iNOS or eNOS was not altered by pre-incubation with SSc serum for 24 h in the cell medium (Fig. 5a).

Likewise, SSc serum did not induce detectable change in the levels of iNOS or eNOS mRNA expression in endothelial cells (Fig. 5b).

Endothelial cell viability

Since endothelial NOS activity and NO production were reduced by the presence of SSc serum in the cell medium, the effect of patient serum on cell survival was assessed. Endothelial cells incubated with SSc serum for up to 48 h showed no decline in cell viability compared with controls (Fig. 6a). Dermal fibroblasts (FIBROBLASTS) also showed no difference after patient serum incubation on survival (Fig. 6b).

Discussion

In the present study NO metabolism was classified in relation to the disease of SSc with its different subsets and primary RP.
Patient plasma was measured for: (i) stable NO degradation production: nitrate and nitrite; (ii) nitration production: protein-bound 3-nitrotyrosine and (iii) the endogenous NO inhibitor ADMA. The results show that plasma NO is elevated in primary RP and lSSc, while in dSSc the levels were normal. A novel finding is that elevated levels of circulating nitrated proteins and ADMA were found only in those patients with dSSc compared with healthy controls and other forms of SSc or RP.

We also investigated whether, conversely, factors in SSc serum could induce dermal microvascular endothelial dysfunction in healthy controls. The effects of patient serum were measured on: (iv) cellular NO synthase activity, (v) intracellular cGMP production and (vi) total nitrate production. The findings show that the nature of the scleroderma serum initially reduces NO activity and production, but not expression of the NOS isoforms.

NO has recently been proposed to be a double-edged sword in SSc and other diseases [14, 15]. Vasospasm and cold-induced digital ischaemic attacks are in part attributed to reduced levels of NO; however, in inflammatory disorders an enhanced cytokine profile (as evident in SSc) may result in NO over-production and subsequent free-radical-mediated tissue damage [14, 15, 20, 21]. Several groups have measured NOx production and apparent contradictory results have emerged [8–12]. The discrepancy in these results could be explained by differences in the degree of inflammatory disorder, disease subset and treatment of the patients. Furthermore, modifications in the dietary NOx intake were not attempted in many of these earlier studies. In order to minimize the effect of the diet, subjects for NOx plasma measurement in this study were maintained on a low-nitrate/nitrite diet for 3 full days, and then fasted overnight. SSc patients were also further differentiated into limited and diffuse disease subsets. Our observation of increased levels of circulating nitrate/nitrite indicates increased NO formation, which is consistent with inflammatory and immunological involvement in the disease
normal controls and late-stage SSc patients as opposed to early stages. Cell survival was assessed in (a) human dermal microvascular endothelial cells (HMEC; n = 4 ISSc, n = 4 dSSc) or (b) the human dermal fibroblast cell line (FIBROBLASTS; n = 6 ISSc, n = 6 dSSc) using the MTT assay as described in the methods. Data (mean ± s.e.m) were expressed as a percentage of the control.

Fig. 6. Effect of serum from SSc patients and normal controls on cell survival. Cells were pre-incubated with control or SSc serum (20% in MCDB 131 medium) for up to 48 h. Cell survival was assessed in (a) human dermal microvascular endothelial cells (HMEC; n = 4 ISSc, n = 4 dSSc) or (b) the human dermal fibroblast cell line (FIBROBLASTS; n = 6 ISSc, n = 6 dSSc) using the MTT assay as described in the methods. Data (mean ± s.e.m) were expressed as a percentage of the control.

of primary RP and ISSc [1, 4]. Other groups [10, 12, 35, 36] also demonstrated an increase in NO formation. The enhanced levels of nitrate were consistent with the increased levels of cGMP in the urine [12], suggesting that the increased formation of NO activates guanylate cyclase.

Further evidence supporting increased NO production in SSc has come from the immunohistological studies on skin which showed increased iNOS expression in SSc tissue [10, 11]. Other studies have also reported NO over-expression in skin SSc fibroblasts [36], and an association with increased endothelial activation in skin SSc biopsy specimens [12]. Indeed, in an elegant series of experiments Cotton and co-workers [11] have demonstrated in the SSc microvasculature an enhanced expression of iNOS associated with a down-regulation of eNOS and proposed a switch in expression of these NOS isoforms. Several observations of impairment in endothelial NOS in SSc support this proposal [8, 9, 37, 38]. It is possible that stimulation of iNOS by inflammatory cytokines may outweigh the effects of eNOS impairment, which is observed in primary RP and SSc patients [9, 11].

Furthermore, in the present study it was noted that nitrate levels in dSSc patients are similar to controls and may indicate that the influences regulating NO production are time dependent. A similar observation has been reported by Takagi and colleagues [36] where there was no significant difference in serum NOx levels between normal controls and late-stage SSc patients as opposed to early stage SSc patients. Additionally, Cotton and co-workers [11] have also demonstrated that although there is a strong staining of iNOS in patients with low grades of skin fibrosis, this declined in the patients with high grades of skin fibrosis.

In a number of inflammatory disorders, NO production from iNOS is increased and reacts with superoxide anions to form peroxynitrite. There is a growing literature showing strong tissue localization of nitrotyrosine staining or increased levels of free and protein-bound nitrotyrosine in inflammatory diseases. For example, strong staining of nitrotyrosine has been found in lung sections from patients with lung injury [39], and in skin sections in patients with SSc [11]. Significant amounts of 3-nitrotyrosine have been reported from patients with rheumatoid arthritis [40] and chronic renal failure [41]. The present data show that in plasma, protein-bound 3-nitrotyrosine was significantly increased in dSSc. This is the first time to our knowledge that a rise in nitrated plasma proteins has been reported in SSc. This has been shown to occur in chronic renal disease in children [42], patients undergoing long-term haemodialysis with diabetes [43] and cardiovascular disease [44]. Furthermore, this increase is strongly inversely associated with the duration of onset of dSSc disease as well as positively with its degree of severity. Enhanced staining of nitrotyrosine in dSSc skin biopsy sections was also observed as previously reported by other groups [11], and was particularly localized around microvessels and the epidermal layer.

The mechanisms behind the elevated concentration of nitrated proteins are unclear. It may be simply explained by an increase in the formation of superoxide anion. Elevated levels of NO were only found in the groups of primary RP and ISSc, but not in dSSc. Monocytes from SSc patients released an excess of superoxide anion [22], particularly in patients with dSSc, at the early stage of onset, a finding that is consistent with the observations reported here. An alternative reason for this specific high level in the group of dSSc is that mechanisms for removal of nitrated proteins may be defective. The presence of nitrating activity has been demonstrated in normal physiological process [45]. There is a growing literature that suggests that protein nitration is a dynamic process rather than an irreversible event [46]. De-nitration of nitrated proteins may decrease peroxynitrite toxicity and restore the normal function of proteins. The existence of a ‘nitrotyrosine de-nitrase’ has been proposed, though the product and possible cofactors for this reverse reaction have not yet been identified [46]. Nevertheless, the nitration of proteins may alter a protein’s confirmation and structure, catalytic activity or susceptibility to protease digestion [19]. Tyrosine nitration in particular may diminish the availability of proteins as a substrate for tyrosine kinases [47].

In mammalian systems levels of NO are closely regulated by naturally occurring endogenous inhibitors such as ADMA [16, 17]. Growing interest in ADMA has revealed elevated levels in diabetes, atherosclerosis, hypercholesterolemia and other associated cardiovascular and non-cardiovascular conditions [17]. In the present study a significantly increased level of circulating ADMA in dSSc patients was shown, while in ISSc levels were elevated but not significantly different from controls and primary RP. This observation supports our findings of lower NOx levels in the dSSc subgroup compared with RP or ISSc. This is a novel finding, with the exception of Rajagopalan and co-workers [18] who recently demonstrated enhanced ADMA in secondary RP as opposed to primary RP patients. Indeed, other studies have suggested that under conditions when NO over-production occurs, S-nitrosylation of the ADMA regulating enzyme dimethylarginine dimethylamino-hydrolase (DDAH) diminishes DDAH activity, leading to an accumulation of ADMA. Subsequently, NOS inhibition as a type of regulatory feedback mechanism may result [48]. The findings presented in this study have demonstrated the presence of a consistent abnormal NO metabolism in the plasma of RP, ISSc and dSSc patients that may
reflect the underlying endothelial damage and vasculopathy of these diseases.

Endothelial dysfunction in SSc has been considered to be among the primary events during the progression of the disease [2, 3, 8]. For example, there is evidence of reduced intracellular eNOS production in the SSc endothelium [8, 9, 37, 38], and increased endothelial activation [12]. Increased endothelial apoptosis mediated by anti-endothelial cell antibodies and antibody-dependent cell cytotoxicity has also been shown to precede inflammatory events and fibrosis [3]. While the aetiology of endothelial dysfunction is still unclear, free-radical-mediated damage and immunological insults remain attractive proposals to mediate effects. In the present study, the extent to which factors present in serum from patients with SSc could affect NO metabolism in normal endothelial cells was investigated. The findings reveal that SSc serum significantly reduces NO synthase activity, paralleled by decreases in intracellular cGMP and NO production in the cell medium, and suggest the presence of a factor that inhibits NOS. These results support the observations from earlier studies that serum factors influence endothelial dysfunction and apoptosis; however, it has been reported that SSc serum alone was ineffective and the additional presence of natural killer cells was required to mediate cytotoxicity in particular [3, 49, 50]. In the work reported here it is also demonstrated that SSc serum alone had no significant effect on endothelial cytotoxicity and the observations of reduced NO activity and production were unlikely to be due to decreased cell survival. While free-radical-related mechanisms and immunological insults evidently mediate endothelial dysfunction these findings also suggest the role of circulating inhibitory factors such as ADMA that may regulate NOS activity in vivo, and account for the observed serum induced decrease in cGMP and NO production in vitro.

In conclusion, formation of NO is increased in patients with primary RP or limited scleroderma, but nitration of proteins and ADMA is a particular feature of diffuse scleroderma and may reflect abnormal NO regulation and/or contribute to endothelial dysfunction in SSc.

Acknowledgements

We gratefully acknowledge the financial support of the Arthritis Research Campaign.

The authors have declared no conflicts of interest.

References


