CONCISE REPORT

Low plasma protein nitrotyrosine levels distinguish primary Raynaud's phenomenon from scleroderma

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Objective: To investigate the hypothesis that increased formation of reactive nitrogen species may contribute to the vascular pathology that develops in patients with connective tissue disease such as scleroderma.

Patients and methods: The level of protein-bound nitrotyrosine in plasma was measured by stable isotope dilution gas chromatography/negative ion chemical ionisation mass spectrometry in 11 patients with primary Raynaud's phenomenon, 37 with scleroderma, 13 with chronic renal impairment, and in 23 healthy controls.

Results: Plasma protein-bound nitrotyrosine was markedly decreased in patients with primary Raynaud's phenomenon (mean (SEM) 0.60 (0.06) ng/mg dry protein) compared with patients with scleroderma (1.78 (0.21) ng/mg protein), chronic renal impairment (1.42 (0.17) ng/mg protein) or healthy controls (1.63 ± 0.15 ng/mg protein, ANOVA $p < 0.001$).

Conclusion: These data suggest that there is decreased nitration of plasma proteins, or increased degradation of nitrated proteins from the circulation of patients with primary but not secondary Raynaud's phenomenon.

Raynaud's phenomenon is commonly associated with connective tissue disorders, notably scleroderma (systemic sclerosis) but also systemic lupus erythematosus, polymyositis, and others. It also occurs frequently in otherwise healthy subjects (primary Raynaud's phenomenon) when the symptoms are generally milder and more responsive to vasodilator treatment. Although almost all patients with scleroderma manifest secondary Raynaud's phenomenon, only a minority develop systemic features of scleroderma, suggesting that the pathogenic mechanisms and structural vascular consequences of primary and secondary Raynaud's phenomenon are distinct.

Patients with Raynaud's phenomenon have abnormal digital vasoconstriction, which may be secondary to impaired synthesis of nitric oxide, and studies have suggested that topical application of a nitric oxide donor may improve the microcirculation of patients with Raynaud's phenomenon.¹ Scleroderma is a heterogeneous multisystem disorder that involves inflammatory, vascular, and fibrotic pathology. Cutaneous fibrosis and Raynaud's phenomenon are almost always present. Recent studies have shown increased lipid peroxidation products in both diffuse and limited cutaneous subsets of scleroderma,² and it is now recognised that reactive nitrogen species, including peroxynitrite, contribute to oxidative and nitrative stress (nitrooxidation) in a variety of clinical disorders. The formation of reactive nitrogen species such as peroxynitrite can be inferred by quantification of the levels of nitrated tyrosine residues (nitrotyrosine) in proteins.

This study aimed to measure and compare the nitrotyrosine content of circulating plasma proteins in patients with primary or secondary Raynaud's phenomenon and a group of patients with impaired renal function, and healthy controls.

PATIENTS AND METHODS

Samples were obtained from randomly selected patients with primary Raynaud's phenomenon, scleroderma, chronic renal impairment, and healthy controls, after receiving informed consent. The diagnosis of primary Raynaud's phenomenon was based on a detailed history and/or observation of bilateral, cold-induced blanching and/or cyanosis of the fingers, or fingers and toes in patients who had no clinical features of connective tissue disease, antinuclear antibodies or antibodies to extractable nuclear antigens or abnormal digital nailfold capillaroscopy.³ All patients with scleroderma satisfied the American College of Rheumatology preliminary classification criteria and were categorised into two subgroups of diffuse or limited cutaneous scleroderma.⁴ Because many patients with scleroderma have impaired renal function, patients with stable impaired renal function were selected from outpatient departments to serve as an additional control group. The glomerular filtration rate was calculated using an equation developed in the Modification of Diet in Renal Disease study.⁵

Protein-bound tyrosine and nitrotyrosine were measured by stable isotope dilution gas chromatography/negative ion chemical ionisation mass spectrometry, as previously described.⁶ This method avoids the introduction of artefactual nitration of proteins during investigation that is inherent in some methods. Results were compared using analysis of variance (ANOVA).

RESULTS

Nitrotyrosine levels are expressed as either the absolute amount per mg of dry protein, or as a ratio to tyrosine. Expression as a ratio reflects the percentage of tyrosine residues in protein that are nitrated. Table 1 shows patient characteristics and the plasma protein nitrotyrosine/tyrosine ratios for the patients and control groups. Figure 1 shows the plasma protein nitrotyrosine levels for each of the groups.

Plasma protein nitrotyrosine levels were significantly decreased in patients with primary Raynaud's phenomenon compared with patients with scleroderma, chronic renal impairment or healthy volunteers (mean (SEM) 0.60 ± 0.06 ng/mg *v* 1.78 (0.21), 1.42 (0.17), and 1.63 (0.15), respectively) (fig 1, ANOVA $p < 0.001$). The plasma protein nitrotyrosine/tyrosine ratio was also significantly reduced in patients with primary Raynaud's phenomenon compared with all non-Raynaud's subjects (table 1, ANOVA $p < 0.001$). The levels of protein-bound tyrosine were similar in each of the five groups (data not shown). There were no statistically significant differences between plasma protein nitrotyrosine or plasma protein nitrotyrosine/tyrosine ratios

Table 1 Patient characteristics, mean glomerular filtration rate (GFR), and the plasma protein nitrotyrosine/tyrosine ratio

	No	Age Mean (range)	Sex (M:F)	Smokers	Mean (SEM) GFR (ml/min)	Mean (SEM) protein nitrotyrosine/tyrosine ratio (pg/μg)
Healthy controls	23	31 (21–50)	10:13	5	90 (3)	24.8 (2.2)
Chronic renal impairment	13	44 (22–88)	6:7	4	66 (8)	21.9 (2.4)
Diffuse cutaneous scleroderma	12	56 (31–77)	1:11	0	70 (6)	31.7 (7.2)
Limited cutaneous scleroderma	25	53 (33–74)	5:20	5	78 (6)	29.1 (5.9)
Primary Raynaud's phenomenon	11	54 (38–70)	2:9	1	86 (5)	10.6 (1.0)

between patients with scleroderma, chronic renal impairment or controls. Intragroup comparisons of plasma protein nitrotyrosine or plasma protein nitrotyrosine/tyrosine ratios between subsets of the scleroderma group did not identify significant differences (table 1). There was no correlation between plasma protein nitrotyrosine or plasma protein nitrotyrosine/tyrosine ratio and the duration of scleroderma, maximum skin score, calculated glomerular filtration rate or history of smoking.

DISCUSSION

This study demonstrates a striking reduction in plasma protein nitrotyrosine in patients with primary Raynaud's phenomenon in comparison with patients with scleroderma, and control groups. This finding was unexpected, particularly because virtually all patients with scleroderma also have Raynaud's phenomenon. Others studies have identified nitrotyrosine by immunohistochemistry in scleroderma skin.⁷ The mean plasma protein nitrotyrosine in our sample of patients with scleroderma, who tended to have more severe and more frequent episodes of Raynaud's phenomenon, was not significantly different from that of the control groups. However, the highest plasma nitrotyrosine values seen in this study were identified in the scleroderma group, suggesting that some patients have increased formation of reactive nitrogen species.

A number of mechanisms might account for our observations. Firstly, there may be decreased formation of nitric oxide and reactive nitrogen species in patients with primary Raynaud's phenomenon. However, previous studies have suggested that plasma nitrite/nitrate concentrations are normal in patients with primary Raynaud's phenomenon.⁸ Secondly, because protein nitration is a dynamic process and nitrated proteins are degraded more rapidly than normal proteins,^{9,10} there may be up regulated degradation or denitration of nitrated proteins in patients with primary Raynaud's phenomenon. Possibly, reduced levels of nitrotyrosine are a significant factor in determining the vascular consequences of Raynaud's phenomenon and the low levels seen in primary Raynaud's phenomenon are protective. If so, then strategies that aim to reduce plasma nitrotyrosine levels in cases of severe secondary Raynaud's phenomenon may abrogate the effects of longstanding Raynaud's phenomenon in connective tissue disease.

These data suggest that decreased formation of reactive nitrogen species or increased degradation of nitrated proteins may be important in the pathogenesis of primary Raynaud's phenomenon, and further suggest that there is a distinct difference between this group of patients and patients with scleroderma who also exhibit Raynaud's phenomenon. Further studies are needed to elucidate some of the mechanisms involved.

Figure 1 Protein-bound nitrotyrosine is decreased in patients with primary Raynaud's phenomenon. Plasma protein-bound nitrotyrosine was measured in 23 healthy controls, 13 patients with chronic renal impairment (CRI), 37 with scleroderma (SSc), and 11 with primary Raynaud's phenomenon.

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