Nerve growth factor and neuropeptides circulating levels in systemic sclerosis (scleroderma)

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Abstract

Objective—To determine the circulating levels of nerve growth factor (NGF), neuropeptide Y (NPY), and vasoactive intestinal peptide (VIP) in systemic sclerosis (SSc), and to correlate these levels with clinical and laboratory features.

Methods—Forty four patients with SSc were evaluated for circulating NGF (immunoenzymatic assay), NPY and VIP (radioimmunoassay), anticientromere and antitopoisoisomerase I autoantibodies, lung disease (pulmonary function tests with carbon monoxide transfer factor (TLCO), ventilation scintiscan with 99mTc DTPA radioaerosol, high resolution computed tomodraphy (HRCT), pulmonary pressure (echo colour Doppler)), heart disease (standard and 24 ECG, echocardiography), cutaneous involvement (skin score), joint involvement (evidence of tender or swollen joints, or both), peripheral nervous system (PNS) involvement (electromyography), rheumatoid factor, angiotensin converting enzyme (fluorimetric method), von Willebrand factor (ELISA), and erythrocyte sedimentation rate (ESR) (Westergren).

Results—Circulating NGF levels in SSc were significantly increased compared with controls (p<0.00001) and significantly higher in the diffuse than in the limited subset of patients (p<0.01). Patients with articular disease had significantly higher levels of NGF. A significant indirect correlation between NGF levels and TLCO was detected (p<0.01), but no correlation was found between NPY and HRCT, DTPA, skin score, PNS involvement, and angiotensin converting enzyme and von Willebrand factor levels, antitopoisoisomerase or anticientromere antibodies, and ESR. NGF levels increased progressively as the disease worsened. Similarly, VIP circulating levels were increased in patients with SSc (p<0.001), whereas the increase of NPY levels did not reach statistical significance. However, both neuropeptides, following the same trend as NGF, increased as the disease worsened (skin score and lung disease).

Conclusions—The increase of NGF and VIP in patients with SSc, the former in the diffuse subset of the disease, and in patients with prominent articular disease, may suggest a link between neurotransmitters and the disease pathogenesis.

Neuropeptide circulating levels seem to increase only in patients with the most severe disease.

(Systemic sclerosis (SSc) is a disease which affects both the microvasculature and the connective tissue. The pathogenesis is characterised by immune abnormalities, endothelial injury, and activation of fibroblasts with consequent collagen accumulation, leading to fibrosis of the skin and internal organs (lung, heart, kidney, gut). An increasing body of evidence indicates that the peripheral nervous system (PNS) is often affected in SSc, though it is not clearly understood whether the involvement represents a primary or a secondary event. None the less, PNS involvement is important in disease pathogenesis, because it has a pivotal role in the dysregulation of vascular tone, which is one of the main features, in particular in the earliest phase, of the disease.

Data generated in the past two decades have defined the properties and characteristics of nerve growth factor (NGF). NGF is a neurotrophic factor with a bioregulatory function on the nervous system and morphogenic, endoautoparacrine, and immunomodulatory functions. NGF seems to be synthesised by the peripheral terminals of the sympathetic and sensory neurons followed by NGF retrograde axonal transport. This mechanism can be blocked by capsaicin and antibodies specific to NGF. In the skin, NGF is produced by fibroblasts, mast cells, lymphocytes, and keratinocytes. NGF may act as controller of cutaneous morphogenesis, pigmentation, wound healing, and inflammation. Indeed, in the PNS, NGF modulates the function of the nerve terminals, controlling their development, survival, and both the production and release of neurotransmitters. Increased amounts of NGF have been found in several autoimmune diseases. Moreover, NGF levels correlate with disease activity in vasculitides and in inflammatory joint diseases in adults and in children. In a previous study, one of us (LA) showed a specific NGF immunoreactivity in SSc dermis in association with high numbers of mast cells.

Neuropeptides are produced and released mainly by the PNS, but they may also be produced directly by both the immune and the endothelial system. Neuropeptides exert a wide control on the microenvironment, either...
enhancing or inhibiting different cellular functions and NGF may modulate their release and action.19 20 In SSc, previous studies have detected raised levels of neuropeptides—namely, substance P24 and calcitonin gene

Table 1 Values of clinical and laboratory parameters in patients with systemic sclerosis

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Vasoactive intestinal peptide (pg/ml)

![Figure 1](A) Serum levels of nerve growth factor (NGF) (ng/ml) in patients with systemic sclerosis (SSc) and controls (C), *p<0.00001; (B) NGF levels (ng/ml) in limited (dSSc) and diffuse (dSSc) subsets of patients with SSc, *p<0.01; (C) NGF levels (ng/ml) in patients with SSc with joint involvement (JI) and without joint involvement (WJI), *p<0.00002.

![Figure 2](Vasovactive intestinal peptide levels (pg/ml) in the sera of patients with systemic sclerosis (SSc) and controls (C), p<0.001.)

Our work aimed at determining the levels of NGF, NPY, and VIP in a larger cohort of patients with SSc, and correlating these levels with clinical, functional, and laboratory features.

Patients and methods

PATIENTS

Forty four consecutive patients with SSc (26 with the limited and 18 with the diffuse subset of the disease)26 were enrolled in this study. Forty two were women and two were men with a mean age of 51.5 years and mean disease duration of 11.3 years. All the patients were receiving the following drugs: calcium channel blockers, proton pump inhibitors, cisapride, topical glyceryl trinitrate. At the moment of sampling the patients were not receiving cyclophosphamide or other disease modifying drugs. Forty healthy volunteers, matched for sex and age, served as control subjects. After signed consent, blood samples were drawn from the antecubital vein of all patients in the related peptide, in the early phase of the disease as well as a decrease in the advanced phase of the disease.23 On the other hand, normal circulating levels of other neuropeptides—namely, vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY), have been detected in studies on a small number of patients with SSc.24 25

For a list of authors, see www.annrheumdis.com
morning between 8:00 and 9:00 am and immediately spun in a refrigerated centrifuge. The supernatants were collected and stored in aliquots at −70°C.

NGF ASSAY
The levels of NGF were measured by a modified sensitive two-site immunoenzymatic assay which recognises human and murine NGF and does not cross react with brain derived neurotrophic factor. Briefly, polystyrene 96-well immunoplates (Nunc) were coated with monoclonal mouse anti-NGF (Boehringer Mannheim, Germany) diluted in 0.05 M carbonate buffer (pH 9.6). Parallel wells were coated with purified goat IgG (Zymed, San Francisco, CA, USA) for evaluation of the non-specific signal. After overnight incubation at room temperature and two hours’ incubation with a blocking buffer (0.05 M carbonate buffer, pH 9.5, 1% bovine serum albumin (BSA)), plates were washed three times with 50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 0.5% gelatin, 0.1% Triton X-100. The samples were collected and centrifuged at 8500 g for 30 minutes. After extensive washing of the plates, the samples and the NGF standard solutions were diluted with sample buffer (0.1% Triton X-100, 100 mM Tris-HCl, pH 7.2, 400 mM NaCl, 4 mM EDTA, 0.2 mM phenylmethylsulphonylfluoride, 0.2 mM benzethonium chloride, 2 mM benzamidine, 40 U/ml aprotinin, 0.05% sodium azide, 2% BSA, and 0.5% gelatin), distributed in the wells, and left at room temperature overnight. The plates were then washed three times and incubated with 4 mU/well anti-β-NGF-galactosidase (Boehringer Mannheim) for two hours at 37°C and, after further washing, 100 µl of substrate solution (4 mg/ml of chlorophenol red (Boehringer Mannheim), substrate buffer: 100 mM HEPES, 150 mM NaCl, 2 mM MgCl₂, 0.1% sodium azide, and 1% BSA) was added to each well. After incubation for two hours at 37°C, the optical density was measured at 575 nm with an enzyme linked immunosorbent assay (ELISA) reader (Dynatech 5000, PBI Int, Germany), and the values of standards and samples were corrected by taking into consideration the non-specific binding. The recovery of NGF during the assay was estimated by adding a known amount of highly purified NGF to the samples or to the homogenisation buffer, as internal control. The yield of exogenous NGF was calculated by subtracting the amount of endogenous NGF from the value of endogenous plus exogenous values. Under these conditions, the NGF recovery was over 90%. The limit of sensitivity of the NGF ELISA was on average 0.5 pg per assay. Data are represented as ng/ml and all assays were performed in triplicate.

NEUROPEPTIDES
The concentration of NPY and VIP was measured with a standard competitive 125I radioimmunoassay kit (Peninsula, Belmont, CA, USA) as previously reported.

AUTOANTIBODIES
Anticentromere and antitopoisomerase I autoantibodies were detected as described elsewhere.

VISCERAL, CUTANEOUS, ARTICULAR DISEASE AND LABORATORY ASSESSMENT
Lung disease was evaluated by pulmonary function tests: vital capacity, forced expiratory volume in one second/forced vital capacity ratio, maximal-minimal expiratory flow rate (measured between 25% and 75% of total vital capacity), carbon monoxide transfer factor (TLCO), ventilation scintiscan with ¹²⁴⁰ Tc DTPA radioaerosol, and high resolution computed tomography (HRCT). Pulmonary pressures were indirectly evaluated by echo colour Doppler. Heart disease was evaluated by standard ECG, 24 hour ambulatory ECG, and echocardiography (M-2D mode and Doppler). When one of these parameters was found to be abnormal, the patient was described as positive.

Cutaneous involvement was assessed by skin score. Joint involvement was recorded as positive when there was objective evidence of tender or swollen joints, or both, as previously reported.

Rheumatoid factor was tested with latex agglutination, and a titre of 1/80 was considered positive. Angiotensin converting enzyme (ACE) and von Willebrand factor (vWF) circulating levels were measured as an index of endothelial derangement. The erythrocyte sedimentation rate (ESR) was evaluated as an acute phase reactant (Westergren).

PNS ASSESSMENT
PNS was assessed by neurophysiological studies. The sensorimotor nerve conduction studies of median, ulnar, posterior tibial, peroneal, and sural nerves were conducted using standard commercial equipment and surface electrodes (Reporter, Esa Ote-Biomedica, Florence, Italy). The technique was performed as previously reported. Any alteration in the assessed tests rendered the patient positive.

ANALYSIS OF DATA: STATISTICAL AND DATA MINING PROCEDURES
The data were analysed by complementary non-parametric statistics. Wilcoxon’s two tailed test was used to analyse and compare non-normally distributed data, which are expressed as median (range). Spearman’s correlation coefficient was employed to correlate laboratory and clinical results.

The data were also analysed using the Statistical package S-PLUS 2000 with a data mining technique. This study took advantage of the S-PLUS graphics features to enhance data structure and patterns during the analysis under the control of an expert doctor (MMC). In particular, a multiparametric visualisation technique, Trellis graphics, was both simple and effective. An example of these as given by S-PLUS can be seen in figs 3A, B, C, 4A, B, and C, 5A, B, and C.

As shown, each graph consists of a series of panels showing an x-y plot conditioned by a chosen variable. For example the sequence of...
four panels reported in fig 5C shows as conditioning the NGF density (x axis NGF range, y axis NGF probability density) for different TLCO ranges; an aggregation of patients with higher NGF emerges. In other words, the online interaction driven by the data, but controlled by an expert, allowed a sequence of logically linked results to enhance a significant data structure that otherwise would be hidden.

Some clustering technique was required for data discrimination: the K means method was an appropriate approach.

Results
CLINICAL AND LABORATORY VALUES
Table 1 shows the values of the assessed clinical and laboratory parameters. A significant increase of vWF levels and a decrease of ACE levels were detected in patients with SSc compared with controls (data not shown). Lung disease, as indicated by the pulmonary tests, was the most common visceral alteration together with involvement of the PNS. Thirteen patients showed joint involvement.
NGF VALUES

Circulating NGF levels in SSc were significantly increased compared with healthy controls (61.3 (range 8–271) ng/ml v 8.3 (2.5–16.4) ng/ml respectively, p<0.00001) (fig 1). Furthermore, NGF levels in the diffuse subset were significantly higher than in the limited subset (95 (8–239) ng/ml v 28 (8–165) ng/ml respectively, p<0.01) (fig 1). Patients with PNS involvement, selected according to electromyographic results, did not show NGF levels significantly higher (46 (8–247) ng/ml) than the patients without PNS involvement (40.1 (12–104) ng/ml), but both groups had significantly higher NGF levels than the control group. Although the highest levels were seen in patients with PNS involvement, this trend did not reach statistical significance.

We noted that 13 patients with joint involvement (seven diffuse and six limited disease) showed significantly higher levels of circulating NGF (117 (39.3–239) ng/ml) than patients without joint disease (20.25 (8–247) ng/ml, p<0.00002) (fig 1).

NEUROPEPTIDES

Circulating VIP levels in patients with SSc were significantly increased compared with healthy controls (18.6 (15.4–31.3) pg/ml v 14.2 (12.7–22.1) pg/ml respectively, p<0.001) (fig 2). On the contrary, no difference was detected in the levels of NPY between patients with SSc and healthy controls.

Figure 4 Data mining elaborations represented by Trellis graphics. In A, B, and C are shown, respectively, the conditioning of the skin score (the range is shown in the x axis, the probability density is shown in the y axis) by neuropeptide Y (NPY), nerve growth factor (NGF), and vasoactive intestinal peptide (VIP). As the skin score increases, a cluster of patients with higher levels of NGF (A) and NPY (B) is identified. This trend is less evident for VIP (C).
controls. No correlation was found between NPY and VIP and the other variables analysed.

**CORRELATIONS AND DATA MINING**

A significant indirect correlation between NGF levels and TLCO was detected (p<0.01, R²=0.00993). No correlation was found between NGF and other neuropeptides and HRCT, DTPA, skin score, PNS involvement, ACE and vWF levels, antitopoisomerase or anticientromere antibodies, and ESR (data not shown).

By the data mining technique the following results were obtained:

- Subset: high NGF and NPY are clustered in the diffuse subset (fig 3)
Nerve growth factor in systemic sclerosis

- Skin score: the increase in the skin score identifies a cluster of patients with the diffuse disease and highest levels of NGF, NPY. This trend is less evident for VIP (fig 4).

- Lung disease: the parameters showing a worsening of the lung function (increase of HRCT score, decrease of TLCO and DTPA) cluster always in the group with diffuse disease with the highest levels of NGF, NPY, and VIP (fig 5).

Analysis of these data shows that the worsening of the disease, in particular in the lungs, is paralleled by an increase of NGF and neuropeptides.

Discussion

Our data clearly demonstrate that NGF levels are strikingly increased in patients with SSc, in particular in the diffuse subset of the disease, as well as in patients with a prominent joint involvement.

The clear cut increase of NGF levels in patients with SSc with articular disease suggests that NGF production may have a link with the involvement of joints, even though no correlation has been shown with ESR as in rheumatoid arthritis and vasculitides. It has been suggested that NGF is a mediator of the acute phase response, and that it plays a part in the development of visceral inflammation. NGF accumulates at the site of inflammation displaying a potent chemoattractant activity for neutrophils and inducing activation of eosinophils. Furthermore, proinflammatory cytokines, such as tumour necrosis factor α and interleukin 1, may vigorously stimulate NGF production, leading to an early and maintained increase of NGF during inflammation, which might result in an enhanced recruitment of inflammatory cells in the affected tissues. It should be emphasised that in SSc, acute phase reactants do not mirror the activity of the disease. In fact, tissue inflammation (skin, alveoli, etc) is considered the main factor in understanding the disease activity. In this work the presence of NGF in the tissues of patients with SSc was not studied. However, previous data published by one of us (LA) showed that NGF is expressed at high levels in the skin of patients with SSc, together with an increase in the number of mast cells, thus contributing to the inflammatory process and potentially to disease pathogenesis.

The indirect correlation between NGF and TLCO is intriguing, as no data have been reported on the relation between NGF and the respiratory system. In patients with SSc the indirect correlation between NGF and TLCO, seen in patients with SSc, may reflect the inflammatory state of the alveolo-capillary membrane in the lung, leading progressively to tissue fibrosis with impairment of pulmonary function. Analysis of our data shows that patients with the diffuse subset of the disease reach the highest levels of NGF and NGF increases as the lung disease worsens as shown by HRCT, TLCO, and DTPA (fig 5). This behaviour of NGF is also seen in correlation with the worsening of the skin involvement, as shown by the skin score (fig 4).

As far as the plasma levels of the two neuropeptides are concerned, we found that only VIP values increased significantly compared with controls. On the contrary, the increase in NPY levels did not reach statistical significance. However, both neuropeptide circulating levels, in particular VIP, increased progressively with the worsening of the skin involvement (fig 4), in particular in the diffuse subset (fig 3). Conflicting results have been reported about the levels of VIP and NPY in SSc. Two studies found normal plasma levels, not differing from controls, and one study found increased VIP levels after stimulation of oesophageal dysmotility with TENS, in SSc. These data focus the attention on the nervous system as a potential source of neuropeptides, suggesting that a dysfunction and/or a linkage to a derangement of the PNS may lead to a poor production of neuropeptides in the advanced phase of the disease. However, in our patients, the lack of correlation among the dysfunction of the PNS and NGF, VIP, and NPY levels does not allow them to be linked conclusively to the nervous system.

Ischaemia-reperfusion is a common event during SSc and circulating NGF may have a vascular and reperfusion role. NGF is a mediator of oxidative homeostasis, by inducing the production of oxygen free radical scavengers after injury, thus showing a reparative role. It is now clear that NGF reduces injury due to oxidative stress: NGF stimulates glutathione sulphhydryl peroxidase, increases the uptake of cysteine and cystine, and during oxidative stress, it extends the half life of γ-glutamylcysteine synthetase mRNA. NGF is produced rapidly in the myocardium after brief myocardial ischaemia, or exogenously infused and endogenously released NGF protects against postischaemic neural stunning and sympathetic cardiac innervation. But still more convincing data are needed to prove whether, in SSc, NGF is linked to reperfusion injury.

In conclusion, we clearly show that NGF levels are increased in SSc and that the highest levels are found in the diffuse subset. Indeed, this work connects for the first time the increase of NGF circulating levels with lung disease and suggest a relation between NGF and neuropeptides. Further studies are needed to understand the relation between NGF and specific organ involvement, thus clarifying its role in the multifaceted pathogenesis of SSc.

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Nerve growth factor and neuropeptides circulating levels in systemic sclerosis (scleroderma)

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