Regulation of substance P mRNA expression in human dermal microvascular endothelial cells

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ABSTRACT

Vascular endothelial cells have been identified as a source of substance P (SP) which may act in an autocrine/paracrine fashion to bring about nitric oxide (NO)-dependent vasodilatation and mitogen-induced cell division or immunologic and inflammatory responses. Whilst SP is localised in and released from endothelial cells, an endothelial mRNA expression of SP has not previously been shown. In the present study, mRNA expression of SP in human dermal microvascular endothelial cells is demonstrated using in situ hybridisation techniques with enhancement procedures. Incubation of microvascular endothelial cells with nerve growth factor (NGF) under conditions of increased shear stress increases the mRNA expression and release of SP. Endothelin (ET) release is also enhanced. These changes are pertinent to circulatory events that may occur in Raynaud's phenomenon in systemic sclerosis.

Introduction

Endothelial cells have been proposed as a source of substance P (SP) that may act in an autocrine/paracrine fashion to affect vascular tone (1, 2). Localisation of SP in endothelial cells has been demonstrated at the electron microscope and light microscope level (1-4), and release of SP from the endothelium has been shown from several blood vessels and from isolated endothelial cells in culture (5, 6). In addition, endothelial cells possess the enzymes, peptidylglycine a-monooxygenase (PAM) and peptidylamidoglycolate lyase (PGL), necessary for formation of the active peptide from their glycine extended precursors (7). However, definitive evidence for the synthesis of SP by endothelial cells has not yet been provided.

Earlier studies describing SP levels in endothelial cells were unable to demonstrate endothelial SP mRNA expression (8). We have recently shown that chronic changes in the innervation profile of blood vessels results in changes in the expression and release of endothelial peptides, including SP and endothelin (ET) (4). It has also been shown that several circulating elements affect the endothelial release of ET (9, 10). It is now well known the pivotal contribution of neuropeptides released by peripheral nervous system to the control of vascular tone. In this perspective, it is still a matter of debate if SP may be produced and released not only by sensory fibers but also by endothelial cells, in particular conditions of stimuli.

It was while discussing these issues with Carwyle E. LeRoy in the early 1990s that for the first time it became clear to two of us (B. Kahaleh, M. Matucci-Cerinic) that the neuropeptides might play an important role in the regulation of vascular tone in the Raynaud's phenomenon associated with a disease such as Systemic Sclerosis (SSc). This prompted us to study the circulating levels of SP in systemic sclerosis (12) and to put forward a hypothesis of dysregulated neuroendothelial control (13). Our aim was to study SP mRNA expression in human dermal microvascular endothelial cells (HDMVEC) and to investigate the effects that shear stress and two stimulating factor such as nerve growth factor (NGF) and ET may have on the expression of mRNA encoding SP.

Materials and methods

Incubation conditions for cells

HDMVEC isolated from adult skin capillaries and cryopreserved at first passage (Cell Applications, Inc., TCS Biologicals, UK) were plated onto coverslips and allowed to grow to conflu-

ence in the presence of M199 culture medium containing 75 µg/ml endothelial cell growth supplement, 15% fetal calf serum, 3.3 x 10^{-5} M isobutyl-methyl-xanthine and 5 x 10^{-4} M dibutyryl-cAMP at 37ºC in a 5% CO_2 atmosphere. The medium was replaced with Dulbecco Phosphate buffer saline supplemented with Ca^{++} and Mg^{++} (DPBS, Sigma,UK) and the HDMVEC were then incubated under zero shear stress, or in a cone and plate apparatus designed in our laboratory (14) under laminar shear stress of 40 dynes/cm^2 with medium alone (DPBS), medium containing NGF (50 ng/ml) (NGF-7S), or medium containing ET (10^{-6} M), for 4 hr at 37ºC. The media were carefully pipetted off so as not to disturb the cells and the plates were stored at -20ºC until assayed for SP and ET levels. The cells were fixed with 4% paraformaldehyde in PBS for 30 min and then washed with PBS merthiolate. Four HDMVEC preparations were investigated and analysed in duplicate, under all conditions.

Determination of SP and ET released from cells

The amount of SP and ET released from the HDMVEC into the media was assayed by enzyme-linked immunosorbent assays, as previously described (2, 5).

In situ *hybridisation – SP mRNA expression in HDMVEC*

The HDMVEC were processed for cellular localisation of mRNA for SP using a HybriProbe kit (Biognostik, Göttingen, Germany) and a double FITC-labelled anti-sense oligonucleotide probe, complementary to bases 350-378 of the total sequence of human SP. Negative controls utilised a similarly labelled 30-base probe (5' TCC TCA GTG TCT CTG TAC GTC AGG TCA AT) with no cross homologies to the human gene encoding SP (Biognostik). After post-hybridization washes (1 x SSC followed by 0.1% SSC, as specified in the kit) and blocking of endogenous peroxidases with 0.3% H_2O_2 in PBS for 30 min at room temperature, the cells were incubated with anti-FITC conjugated to HRP (Boehringer), 7.5 U/ml in PBS, 0.05% Tween 20, 0.1% gelatin for 30 min at room temperature and washed with PBS, 0.05% Tween 20. The signal was enhanced using an Indirect Tyramide Signal Applification system (TSA™ Indirect, NEN Life Science Products, Boston, USA), using biotinyl tyramide and detection with streptavidin conjugated to HRP and DAB-H_2O_2, as specified in the kit instructions.

The coverslips were mounted on glass slides and viewed under a Zeiss microscope. Photographs were taken under identical settings.

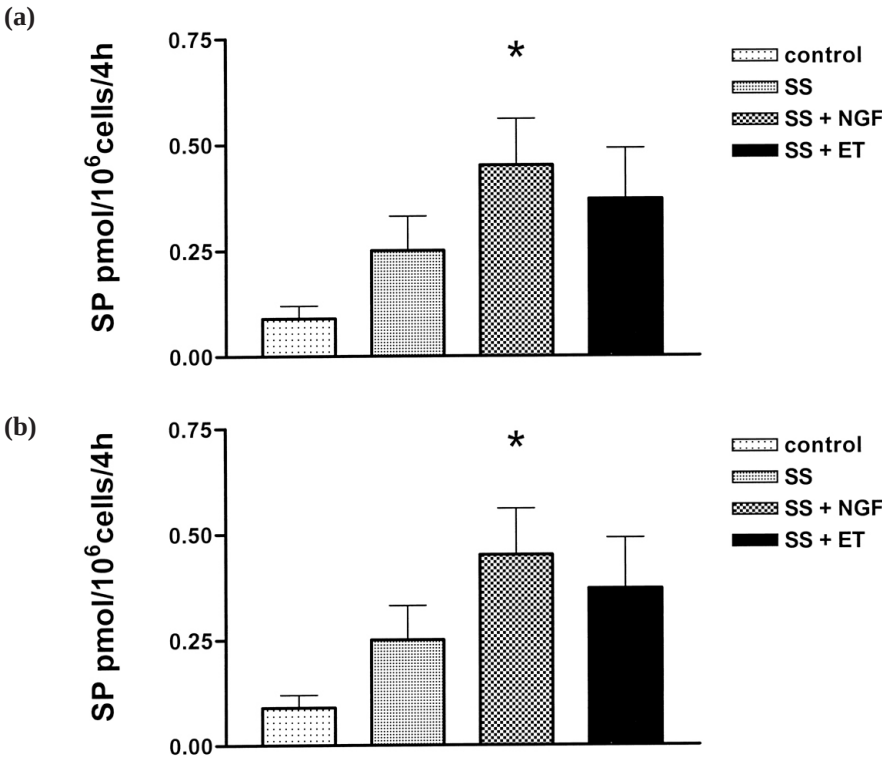

Fig. 1. (**a**) SP released from HDMVEC under conditions of no shear stress (control), 40 dynes/cm^2 shear stress (SS), 40 dynes/cm^2 shear stress and NGF (50 ng/ml) (SS+NGF), and 40 dynes/cm^2 shear stress and ET (10^{-6}M) (SS+ET), expressed as pmol/10^6 cells/4h. n = 4 preparations of HDMVEC, *P < 0.05. (**b**) ET released from HDMVEC in 4 h under conditions of no shear stress (control), 40 dynes/cm^2 shear stress (SS), and 40 dynes/cm^2 shear stress and NGF (50 ng/ml) (SS+NGF), expressed as pmol/10^6 cells/4h. n = 4 preparations of HDMVEC *P < 0.05.

Statistics

The statistical difference between SP and ET released from HDMVEC incubated under the conditions outlined was analysed using Dunnet's Multiple Comparison. A p value of < 0.05 was taken as significant.

Results

SP and ET release from HDMVEC

Incubation of HDMVEC under conditions of increased shear stress (40 dynes/cm^2) in the presence of NGF resulted in a significant increase in the release of SP (0.45 ± 0.11 compared to 0.09 ± 0.03 pmol/10^6 cells/4h, n = 4; p < 0.05) and ET (24.9 ± 7.8 compared to 2.1 ± 0.3 pmol/10^6 cells/4 h, n = 4; p < 0.05) into the media compared with the release from HDMVEC incubated at zero shear stress (Fig. 1). There was a tendency for increased release of SP and ET with increased shear stress alone, although this trend was not significant (Fig. 1). Increased SP release in conditions of increased shear stress in the presence of ET was not significant (Fig. 1).

SP mRNA expression

Positive labelling for SP mRNA expression was observed in the cytoplasm of the HDMVEC that had been maintained at 40 dynes/cm^2 shear stress for 4hr (Fig. 2a). Positive labelling was more prominent in HDMVEC that had been incubated at the same level of shear stress in the presence of either

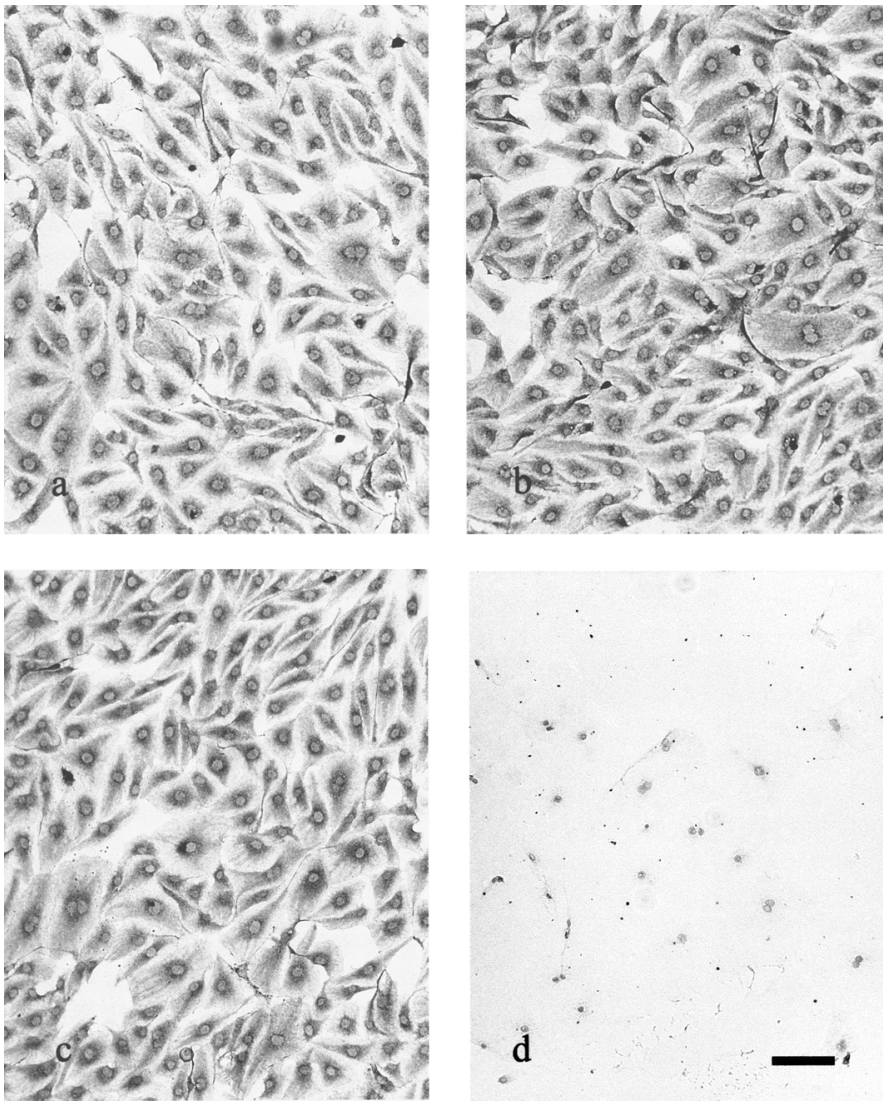

Fig. 2. (**a-c**) Light micrograph of HDMVEC showing positive labelling following *in situ* hybridisation for localisation of SP mRNA expression, with enhancement techniques, following: (**a**) incubation of the cells under shear stress 40 dynes/cm^2; (**b**) incubation of the cells under shear stress 40 dynes/cm^2 in the presence of NGF (50 ng/ml); and (**c**) incubation of the cells under shear stress 40 dynes/cm^2 in the presence of ET (10^{-6} M). Note the more prominent staining in (**b**) and (**c**) than in (**a**). (**d**) shows HDMVEC incubated as in (**b**) but with substitution of the antisense SP probe with a probe with no cross homologies to the gene encoding SP. This negative control shows no staining throughout the cells. Bar = 100μm.

NGF (Fig. 2b) or ET (Fig. 2c). HDMVEC incubated under conditions of increased shear stress and NGF processed with the negative control probe and enhancement procedures showed no staining (Fig. 2d).

Discussion

This is the first demonstration of SP mRNA expression in HDMVEC. Vascular endothelial cells have been identified as a source of SP (1, 15) which may act in an autocrine/paracrine fashion to bring about nitric oxide (NO)-dependent vasodilatation and mitogen-induced cell division or immunologic and inflammatory responses (16). Whilst SP is localised in and released from endothelial cells (1-3), an endothelial mRNA expression of SP has not previously been shown. In the present study, mRNA expression of SP in HDMVEC is demonstrated using *in situ* hybridisation techniques with enhancement procedures. Shear stress was used in order to mimic the conditions that HDMVEC are under *in vivo*. Incubation of HDMVEC with NGF under conditions of increased shear stress upregulates mRNA expression and the release of SP and increases release of ET. An interaction between NGF and endothelial neuropeptide expression has not previously been reported. In the present study, we provide evidence that NGF stimulates SP expression in isolated HDMVEC in the physiological setting of increased shear stress. The increase of SP release from HDMVEC may influence the surrounding microenvironment, in particular inducing vasodilation and extravasation. This event may be clinically evident as tissue edema.

As NGF also stimulates ET release from these cells, the increase of ET may act in an autocrine fashion (17, 18) enhancing NO that supports the vasodilation and edema induced by SP. ET may also balance the system with a vasoconstricting action. It is likely that in pathological conditions SP expression in HDMVEC is significantly enhanced. These experiments demonstrate the complexity of likely ongoing events in human dermal microvasculature. Added to this is the potential enhancement of expression of endothelial SP in response to the loss of sensory innervation around dermal capillaries reported in Raynaud's phenomenon in SSc (13, 15, 20). This suggestion may be reinforced by our previous observation that chronic sensory denervation increases immunoexpression and release of SP from endothelial cells (2, 4).

In conclusion, HDMVEC express and release SP and ET, in particular under NGF stimulation. The fact that NGF increases the expression and production of SP and ET suggests that it may be a pivotal agent in the control of vascular tone. Further studies are warranted to elucidate how interactions between these substances are involved in diseases characterised by dysfunction of vascular tone control such as SSc.

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We dedicate this work also to Dr Philippe Bodin – unique and missed by all.

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