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VCAM-1 Expression on Human Dermal Microvascular Endothelial Cells Is Directly and Specifically Up-Regulated by Substance P

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Sensory nerves in skin are capable of releasing multiple neuropeptides, which modulate inflammatory responses by activating specific cutaneous target cells. Extravasation of particular subsets of leukocytes depends upon the regulated expression of cellular adhesion molecules such as VCAM-1 on microvascular endothelial cells. We examined the direct effect of cutaneous neuropeptides on the expression and function of human dermal microvascular endothelial cell (HDMEC) VCAM-1. A significant increase in VCAM-1 immunostaining of microvascular endothelium was observed in vivo following capsaicin application to human skin. Multiple cutaneous sensory C-fiber-released neuropeptides were evaluated for their ability to induce VCAM-1 cell surface expression on HDMEC. Only substance P (SP) was found to be capable of inducing HDMEC VCAM-1 expression. This SP-mediated VCAM-1 induction appeared to be a direct effect that did not require the release of other HDMEC-derived soluble factors. Increased HDMEC VCAM-1 mRNA expression was detected 1 h after the addition of SP, with peak mRNA increase at 6–9 h postinduction. FACS studies demonstrated a 6.5-fold increase in endothelial cell surface VCAM-1 expression detectable 16 h after addition of SP, which was specifically blocked by a neurokinin-1 receptor antagonist. Increased VCAM-1 cell surface expression on SP-treated HDMEC resulted in a 4-fold increase in the functional binding of 51Cr-labeled MOLT-4 T cells. These data indicate that SP is capable of directly and specifically up-regulating functional endothelial VCAM-1 expression and thus may play a key role in modulating certain inflammatory responses in the skin.


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3 Abbreviations used in this paper: HDMEC, human dermal microvascular endothelial cell(s); SP, substance P; NK-R, neurokinin receptor; NKA, neurokinin A; NKB, neurokinin B; CGRP, calcitonin gene-related peptide; NT, neuropeptide; Soms, somatostatin; NEP, neutral endopeptidase; VLA-4, very late Ag-4.

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cell surface expression of ICAM-1. In the current study, we examine the effect of cutaneous nerve-released neuropeptides on microvascular endothelial cell VCAM-1 expression and function. These studies demonstrate the direct and specific ability of SP to induce VCAM-1 expression and function on these cells, thus further supporting the role of effector neurogenic signals in mediating specific cutaneous inflammatory responses.

Materials and Methods

Cells and reagents

HDMEC isolated from foreskins were obtained from the cell culture facility of the Emory Skin Disease Research Core Center (Atlanta, GA) (18). Procedures were conducted with cells in passages 3–5. HDMEC were cultured on a gelatinized (0.1%) surface in MCDB 131 (Life Technologies, Gaithersburg, MD) supplemented with 10% normal human serum (Irvine Scientific, Santa Ana, CA), 5 ng/ml epidermal growth factor (Clonetics, San Diego, CA), 1 mg/ml hydrocortisone acetate (Sigma, St. Louis, MO), 100 U/ml penicillin, 250 μg/ml amphotericin B, and 10 μg/ml streptomycin (Life Technologies). SV40 large T Ag-transformed HDMEC (HMEC-1) were generated by the Emory Skin Disease Research Core Center (Life Technologies). SV40 large T Ag-transformed HDMEC receptor antagonist GR82334 (Peninsula Laboratories) was added to cultured HDMEC to incubate for 20 min at 37°C at 5% CO2 before the addition of 10 nM SP or TNF-α. Polyclonal rat anti-human SP (American Research Products, Belmont, MA) was diluted to 15 μg/ml in HDMEC medium. The SP-degrading enzyme neutral endopeptidase (NEP) was a gift from Nigel Bunnett (University of California, San Francisco, CA). Lyophilized human recombinant TNF-α and anti-TNF-α Ab were obtained from R&D Systems (Minneapolis, MN). Murine mAb P3C4 directed against human VCAM-1 was a generous gift from Dr. Elizabeth Wayner (University of Minnesota, Minneapolis, MN). Murine mAb 84H10 directed against human ICAM-1, which functions as a blocking Ab was provided by Dr. Stephen Shaw (National Institutes of Health, Bethesda, MD) (20).

In vivo expression of VCAM-1 in human skin microvascular endothelial cells by immunohistochemistry

Capsaicin-high potency (Zostrix, 0.075%, GenDerm, Lincolnshire, IL) was applied topically to the skin of four volunteers to stimulate the release of cutaneous neuropeptides and 4-mm punch biopsies were taken from treated sites at 6, 24, and 48 h as well as from an untreated site on the opposite limb taken immediately before capsaicin application. Tissue was embedded in O.C.T. (Tissue Tek; Miles, Elkhart, IN), frozen, and stored at −70°C. Immunohistochemistry was performed on 8-μm sections using mouse anti-human VCAM-1 mAb P3C4 as the primary Ab (diluted 1:400), a biotinylated horse anti-mouse secondary Ab (Zymed, San Francisco, CA), and strept-avidin-biotin complex (Zymed) as a tertiary reagent to detect specific staining of the primary and secondary reagents. Samples were examined using a Nikon (New York, NY) Microshot SA microscope, and photographed at ×120 magnification using a Nikon NFX-35 microscope. Control staining was performed on tissue processed without the primary Abs. No staining was observed with either of the controls.

ELISA measurement of HDMEC cell surface VCAM-1 expression

HDMEC were plated onto 96-well plates and upon reaching 80% confluence were either left untreated or stimulated for 16 h with 1, 10, 100, or 1000 nM of neuropeptides; SP, NKA, NKB, CGRP, or Sms. Cell viability was determined at the end of the stimulation period by performing cell counts in separate plates. The addition of 300 U/ml TNF-α for 16 h to cultured HDMEC was used as a positive control for VCAM-1 cell surface induction. HDMEC VCAM-1 expression was assessed using the anti-VCAM-1 mAb P3C4 or isotype-matched control IgG by ELISA as described previously (21). Results represent the mean of three values ± SD for each variable tested, and results are representative of three independent assays.
added and allowed to incubate for 15 min at room temperature. Eachwell was then swabbed with two cotton-tipped swabs that were counted in a gamma counter. Adherence was calculated as % MOLT-4 binding = [cpm per well - background cpm]/[cpm added counts - background cpm)] × 100, as described previously (24). Photographs of unlabeled MOLT-4 T cells adhering to untreated or SP-treated HDMEC were taken using Kodak (Rochester, NY) Ektachrome 66 Tungsten film on an OM2 camera (Olympus, New Hyde Park, NY) mounted on an Olympus CK2 microscope.

Statistical analysis
For statistical analysis, Student’s t tests were performed. Values of p > 0.005 were not considered significant.

Results
In vivo induction of VCAM-1 in capsaicin-treated human skin
The in vivo effect of C-fiber-released neuropeptides on dermal endothelial cell VCAM-1 expression was examined. A capsaicin-containing cream (Zostrix) was applied topically to human volunteers to stimulate the release of neuropeptides including SP from cutaneous sensory nerves (25). Biopsies were obtained at 0, 6, 24, and 48 h, and evaluated by immunohistochemistry for VCAM-1 expression associated with microvascular endothelial cells in the dermis. As shown in representative photomicrographs from one individual, microvascular VCAM-1 staining was present in very low levels in untreated skin (Fig. 1a). VCAM-1 immunoreactivity increased slightly 6 h after capsaicin application (Fig. 1b). A significant increase in dermal endothelial cell VCAM-1 staining was detected 24 h after application of topical capsaicin (Fig. 1c), which was decreased in intensity by 48 h after capsaicin application (Fig. 1d). In other subjects, VCAM-1 expression persisted at 48 h after application of capsaicin (data not shown). Biopsies obtained from symmetrical untreated sites showed no increased VCAM-1 staining (data not shown). Thus, the release of neuropeptides by cutaneous sensory nerves results in increased HDMEC VCAM-1 expression in vivo.

Specificity of SP-induced HDMEC VCAM-1 cell surface expression
A number of neuropeptides are detected in normal and inflamed skin (10). The effect of several of these agents known to be released by cutaneous sensory nerves on HDMEC VCAM-1 expression was investigated by ELISA (Fig. 2). Untreated HDMEC (−) have no constitutive VCAM-1 present on their surface. Similarly, treatment with an isotype control IgG in place of the anti-VCAM mAb P3C4 had no effect on HDMEC VCAM-1 surface expression. As indicated, incubation with 1, 10, 100, or 1000 nM SP for 16 h up-regulated cell surface VCAM-1 on HDMEC. The optimal 7-fold induction was observed with the 10 nM SP dose (Fig. 2). In contrast, incubation for 16 h with 1, 10, 100, or 1000 nM of NKA, NKB, NT, CGRP, or Sms had no effect on HDMEC VCAM-1 induction (Fig. 2). Cell viability as determined by cell counts after stimulation was not affected by any of the neuropeptide treatments (data not shown). TNF-α served as a positive control for HDMEC VCAM-1 cell surface induction (Fig. 2).

SP directly induces HDMEC VCAM-1
To test whether SP directly up-regulates the expression of HDMEC VCAM-1 or whether this was in part due to the induction by SP of HDMEC-secreted soluble factors that in turn could be responsible for increased VCAM-1 expression, we performed a series of supernatant transfer experiments (Fig. 3). Initially, we determined that HDMEC treated between 1 and 24 h with 10 nM SP resulted in a maximal 7-fold up-regulation of cell surface VCAM-1 expression at 16 h (Fig. 3A) as measured by ELISA. TNF-α significantly up-regulated VCAM-1 expression compared with untreated cells and served as a positive control (Fig. 3A). Isotype control IgG in place of the anti-VCAM-1 Ab showed no change in VCAM-1 expression as compared with untreated HDMEC (data not shown). The effect of supernatants obtained from SP-treated HDMEC on VCAM-1 expression was measured (Fig. 3, B–D). As indicated in Fig. 3B, conditioned medium from SP-treated HDMEC added to freshly cultured HDMEC for 16 h was capable of augmenting VCAM-1. This was most apparent with supernatants derived from the short SP-HDMEC incubation
FIGURE 3. SP directly up-regulates HDMEC VCAM-1 expression. VCAM-1 expression was measured by ELISA on HDMEC either left untreated (−) or stimulated with 10 nM SP for 1–24 h. A, TNF-α (300 U/ml) treatment of HDMEC for 16 h was used as a positive control for VCAM-1 cell surface induction. The supernatants from the experiments in A were added to freshly cultured HDMEC for 16 h without Ab (B), or with 15 μg/ml anti-SP Ab (C) for 16 h and HDMEC VCAM-1 expression was measured. D, In separate experiments, supernatants from the experiments in A were added to freshly cultured HDMEC with 15 μg/ml anti-SP Ab and 300 U/ml TNF-α for a 16-h incubation period. Statistically significant differences in cell surface VCAM-1 in treated samples as compared with untreated control cells (−) were determined by Student’s t test as indicated by asterisks (*, p < 0.005). The data shown are representative of experiments conducted in triplicate.

The biological consequence of SP-induced HDMEC VCAM-1 expression served following treatment with anti-TNF-α Ab (data not shown). Untreated HMEC-1 were calculated as having a mean channel fluorescence of 1.90 for surface VCAM-1 staining (Fig. 5, dotted lined histograms). HMEC-1 treated with 10 nM SP for 16 h resulted in a >6.5-fold increase in mean fluorescence, with a mean channel fluorescence of 12.73 for surface VCAM-1 (Fig. 5B) compared with control untreated cells. Pretreatment of HMEC-1 with an NK-1R antagonist before the addition of SP resulted in a mean channel fluorescence of 2.26, demonstrating the important role of the NK-1R in SP-induced VCAM-1 cell surface expression, with a mean fluorescence of 45.2 and a 6.5-fold increase in mean fluorescence when exposure to SP (Fig. 4). TNF-α induction of HDMEC VCAM-1 mRNA expression served as a positive control for this study. Thus, SP is capable of inducing HDMEC VCAM-1 mRNA.

SP induces HMEC-1 VCAM-1 cell surface expression

The effect of SP on immortalized HMEC-1 cell surface VCAM-1 expression was examined by FACS analysis and mean log_{10} fluorescence values were compared. Untreated HMEC-1 were calculated as having a mean channel fluorescence of 1.90 for surface VCAM-1 staining (Fig. 5, dotted lined histograms). HMEC-1 treated with 10 nM SP for 16 h resulted in a >6.5-fold increase in mean fluorescence, with a mean channel fluorescence of 12.73 for surface VCAM-1 (Fig. 5B) compared with control untreated cells. Pretreatment of HMEC-1 with an NK-1R antagonist before the addition of SP resulted in a mean channel fluorescence of 2.26, demonstrating the important role of the NK-1R in SP-induced VCAM-1 up-regulation (Fig. 5C). The NK-1R antagonist alone had no effect on VCAM-1 cell surface expression (data not shown). TNF-α-treated HMEC-1 displayed high levels of cell surface VCAM-1 expression, with a mean fluorescence of 45.2 and served as a positive control (Fig. 5D). TNF-α-mediated HMEC-1 VCAM-1 up-regulation was not blocked by pretreatment with an NK-1R antagonist (data not shown). In addition, no increase in fluorescence was observed when an isotype matched control Ab was used on SP-treated HMEC-1 (Fig. 5A). Therefore, these data demonstrate SP up-regulates the expression of cell surface VCAM-1 on HMEC-1, primarily through interaction with the NK-1R.

Enhanced binding of MOLT-4 T cells to SP-treated HDMEC

The biological consequence of SP-induced HDMEC VCAM-1 cell surface expression was determined using a quantitative cellular adhesion assay with MOLT-4 T cells (22). MOLT-4 T cells bear...
Substance P is a neuropeptide released by sensory nerve fibers that can activate multiple cell types. When cutaneous sensory nerves release Substance P (SP), it can directly activate mast cells and keratinocytes to produce cytokines. SP has been shown to modulate leukocyte effector activities such as lymphocyte proliferation, cytotoxicity, and Ig production. SP also induces adhesion molecule expression on endothelial cells, which facilitates leukocyte trafficking to sites of tissue injury. The release of SP can influence leukocyte subset trafficking, including neutrophils, monocytes, and eosinophils.

**Discussion**

Neuroimmunologic interactions leading to activation of endothelial cells and modulation of downstream leukocyte trafficking events may play a key role in regulating inflammatory responses. The release of neuropeptides such as SP by cutaneous sensory fibers may function as effector signals to facilitate increased leukocyte trafficking to sites of infection, inflammation, and wound healing. SP has been well characterized as a potent vasodilator that may function as efferent signals to facilitate increased leukocyte trafficking. Additionally, SP can modulate leukocyte effector activities such as lymphocyte proliferation, cytotoxicity, and Ig production. SP can also induce adhesion molecule expression on endothelial cells, which facilitates leukocyte trafficking to sites of tissue injury. The release of SP can influence leukocyte subset trafficking, including neutrophils, monocytes, and eosinophils.

By the expression of specific cell adhesion molecules, as well as release of cytokines and chemokines, the microvascular endothelium is a key cell in regulating leukocyte trafficking into inflamed tissue. The nature of the inflammatory stimulus determines whether lymphocytes, monocytes, neutrophils, or eosinophils predominate, exercising specificity in molecular signals that control traffic of leukocyte subsets. The main classes of adhesion molecules that play a role in trafficking are the selectins, the integrins, members of the Ig superfamily, and sialoadhesins. In skin, P- and E-selectins mediate initial leukocyte adhesion, whereas ICAM-1 and VCAM-1 mediate subsequent, firm adhesion and transendothelial migration. ICAM-1 binds leukocytes via β2 integrins and can therefore recruit both granulocytes and monocytes from the bloodstream to sites of tissue injury, whereas VCAM-1 binds monocytes, eosinophils, and lymphocytes expressing the integrins α4β1, α6β1, and α5β1, specifically supporting the recruitment of chronic inflammatory cells. SP has been suggested as playing a role in the pathogenesis of psoriasis, where possibly neuropeptides play a role in keratinocyte function, as demonstrated by the benefits of capsaicin treatment.

Our present evaluation of the effect of SP on cutaneous microvascular endothelial cell VCAM-1 expression further supports the role of neuropeptides in the regulation of leukocyte trafficking in the skin. The ability of neuropeptides to influence adhesion molecule expression on microvascular endothelial cells was shown in our recent studies of SP induction of dermal endothelial ICAM-1 expression. Additionally, SP was found to up-regulate adhesins P-selectin and E-selectin in normal human skin when incubated for a limited time period. We demonstrate induction of dermal microvascular endothelial cell VCAM-1 expression in vivo after topical application of the neuropeptide-releasing agent capsaicin. Cutaneous sensory nerve-released neuropeptides NKA, NKB, NT, CGRP, and Sms had no effect on HDMEC VCAM-1 cell surface up-regulation across a broad concentration range, whereas SP significantly increased VCAM-1 cell surface expression.
expression. Additionally, SP was capable of directly up-regulating HDMEC surface VCAM-1, without the participation of other HD-MEC-derived factors. This direct SP induction resulted in increased levels of VCAM-1 mRNA and NK-1-mediated VCAM-1 cell surface expression. SP treatment resulted in functional VCAM-1 induction as demonstrated by the ability of VCAM-1 on HDMEC to bind its ligand VLA-4 on MOLT-4 T cells.

SP is known to induce the secretion of cytokines from various immune cells (32, 35, 41). We determined whether SP exposure resulted in HDMEC secretion of soluble factors capable of inducing VCAM-1 expression. Our study indicates that SP was capable of directly inducing increased HDMEC VCAM-1 expression without the participation of HD-MEC-derived factors. In vivo, cytokine release may play a role in endothelial adhesion molecule up-regulation, but cytokine release is neither necessary nor required for SP-mediated induction of VCAM-1 on cultured microvascular endothelial cells.

In summary, our studies demonstrate that SP is capable of directly regulating dermal microvascular endothelial VCAM-1 expression, primarily through activation of the NK-1 receptor. These findings further support the role of neuropeptide modulation of leukocyte recruitment in the skin during cutaneous inflammatory reactions. The coordination of signals such as neuropeptide release and adhesion molecule up-regulation may play a role in the development of a wide range of inflammatory responses and wound healing. Further investigation into the interactions of various neuropeptides, adhesion molecules, and other inflammatory mediators should provide a rational basis for controlling inflammatory disorders that may have a significant neurogenic component such as rheumatoid arthritis, asthma, psoriasis, and atopic eczema.

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