Neuropeptide regulation of human dermal microvascular endothelial cell ICAM-1 expression and function

KIMBERLY L. QUINLAN, IN-SUNG SONG, NIGEL W. BUNNETT, ELEANOR LETRAN, MARTIN STEINHOF, BRAD HARTEN, JOHN E. OLERUD, CHERYL A. ARMSTRONG, S. WRIGHT CAUGHMAN, AND JOHN C. ANSEL

Department of Dermatology and Emory Skin Diseases Research Core Center, Emory University School of Medicine; Departments of Physiology and Surgery, University of California, San Francisco, California 94140; and Department of Dermatology, University of Washington, Seattle, Washington 98195

Quinlan, Kimberly L., In-Sung Song, Nigel W. Bunnett, Eleanor Letran, Martin Steinhoff, Brad Harten, John E. Olerud, Cheryl A. Armstrong, S. Wright Caughman, and John C. Ansel. Neuropeptide regulation of human dermal microvascular endothelial cell ICAM-1 expression and function. Am. J. Physiol. 275 (Cell Physiol. 44): C1580–C1590, 1998.—There is increasing evidence that sensory nerves may participate in cutaneous inflammatory responses by the release of neuropeptides such as substance P (SP). We examined the direct effect of SP on human dermal microvascular endothelial cell (HDMEC) intercellular adhesion molecule 1 (ICAM-1) expression and function. Our results indicated that, although cultured HDMEC expressed mRNA for neurokinin receptors 1, 2, and 3 (NK-1R, NK-2R, and NK-3R), SP initiated a rapid increase in HDMEC intracellular Ca²⁺ levels, primarily by the activation of NK-1R. Immunohistochemistry studies likewise demonstrated that HDMEC predominantly expressed NK-1R. The addition of SP to HDMEC resulted in a rapid increase in cellular ICAM-1 mRNA levels, followed by a fivefold increase in ICAM-1 cell surface expression. This functionally resulted in a threefold increase in ⁵¹Cr-labeled binding of J-Y lymphoblastoid cells to HDMEC. In vivo studies demonstrated a marked increase in microvascular ICAM-1 immunostaining 24 and 48 h after application of capsaicin to the skin. These results indicate that neuropeptides such as SP are capable of directly activating HDMEC to express increased levels of functional ICAM-1 and further support the role of the cutaneous neurological system in modulating inflammatory processes in the skin.

intercellular adhesion molecule 1; cell surface molecules; neuroimmunology; inflammation; skin

Neurokinin A 4–10) were obtained from Peninsula Laboratories, Belmont, CA. Lyophilized SP was generously provided by Dr. J. Jack Strominger, Dana Farber Cancer Institute, Boston, MA) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.25 mg/ml streptomycin, and 5 mg/ml hydrocortisone acetate (Sigma Chemical, St. Louis, MO), 5 × 10⁻⁵ M dibutyryl adenosine 3',5'-cyclic monophosphate (Sigma), 1 mg/ml epidermal growth factor (Clonetics, San Diego, CA), and 10 µg/ml streptomycin (Life Technologies). Human B lymphoblastoid J-Y cells were cultured in MCDB 131 (Life Technologies, Gaithersburg, MD) supplemented with 10% normal human serum (Irvin Scientific, Santa Ana, CA), 5 ng/ml epidermal growth factor (Clonetics, San Diego, CA), 100 U/ml penicillin, 0.25 mg/ml streptomycin, and 5 mg/ml hydrocortisone acetate (Sigma Chemical, St. Louis, MO), 5 × 10⁻⁵ M dibutyryl adenosine 3',5'-cyclic monophosphate (Sigma), 100 U/ml penicillin, 0.25 mg/ml streptomycin, and 10 µg/ml streptomycin (Life Technologies).

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Determination of neurokinin receptor mRNA expression by RT-PCR. RT-PCR was carried out on untreated HDMEC to measure the expression of NK-1R, NK-2R, and NK-3R mRNA. HDMEC were expanded in culture to 1 × 10⁶ cells and mRNA was harvested via an mRNA isolation kit according to the manufacturer’s instructions (Boehringer Mannheim, Indianapolis, IN). First-strand cDNA was first synthesized using 0.5 μg of oligo(dT)₁₅ as primer, 1 mM dNTPs, 15 units of Moloney murine leukemia virus RT, and 1 μg of mRNA in a reaction volume of 20 μl, according to the manufacturer’s recommended instructions for the Promega reverse transcription system (Promega, Madison, WI). One-twentieth of this reaction product was then subjected to PCR template. Oligonucleotide primers were utilized to amplify a portion of each neurokinin receptor cDNA based on the published sequences (5, 12, 15). The primer sequences were for NK-1R, 5'-CCTCGTCT- GATTTGGCATAG-3' (sense) and 5'-CTCTGGCTCTCTCTC- GTGGG-3' (antisense); for NK-2R, 5'-GCCCTCCTCAGT- GCTCTCTA-3' (sense) and 5'-GGGGGACCAACATCATA-3' (antisense); and for NK-3R, 5'-TCTTCTGGCTCTGCACAG-3' (sense) and 5'-GTTGCTCTTTTTCGACT-3' (antisense). The predicted PCR amplification products of 487, 687, and 655 bp were generated from HDMEC cDNA templates as fragments of NK-1R, NK-2R, and NK-3R, respectively. The amplification profile involved a step for denaturing of the template with 1× PCR buffer for 5 min at 95°C, followed by the addition of 200 μM each dNTP, 1.0 μM each primer, 0.5 U/100 μl Taq polymerase, and 1 pmol/μl sense and antisense primers with 1× PCR buffer and then 35 cycles of amplification, each composed of denaturation at 94°C for 10 s, primer annealing for 10 s (at 58.1°C for NK-1R, 59.3°C for NK-2R, and 53.1°C for NK-3R), and polymerase extension at 72°C for 10 s.

Measurement of intracellular Ca²⁺. Cultured HDMEC were detached by a brief exposure to cell dissociation buffer, an enzyme-free PBS-based buffer (Life Technologies). Cells were then washed twice with PBS(−) (no Ca²⁺ or Mg²⁺), and fura 2-AM (Molecular Probes, Eugene, OR) was incorporated into 3–5 × 10⁵ cells by its addition to 5 ml of 10⁶ cells suspension at a final concentration of 5–10 μM and incubated for 45 min at 37°C. After three washes with PBS(−) buffer, ~6 × 10⁶ cells/ml were transferred to a quartz cuvette and stirred continuously. The cells were then treated with SP alone or were pretreated for 20 min with 1,000 nM neurokinin receptor antagonists (NK-1R, NK-2R, or NK-3R antagonist, Peninsula Laboratories), followed by treatment with 100 nM SP. The neurokinin receptor antagonists were used singly or in combination, as indicated. Fluorescence was recorded in a model LS50 spectrophuorometer (Perkin-Elmer, Branchburg, NJ), with excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. From the ratio of measured fluorescence at the two excitation wavelengths, the intracellular free Ca²⁺ concentration was calculated as described (39). As controls for each experiment, fluorescence was also measured after treatment of the cell suspension with Triton X-100 (0.3%) to lyse the cells and generate maximum Ca²⁺ flux and after treatment with EGTA (10 mM) to chelate Ca²⁺ and abrogate the response.

Determination of in vivo neurokinin receptor expression in microvascular cells by immunohistochemistry. Primary antibodies were raised in rabbits to peptide fragments of NK-1R, NK-2R, and NK-3R conjugated to keyhole limpet hemocyanin, as recently described (14). Punch biopsies obtained from normal skin of volunteers were immediately frozen in liquid nitrogen, embedded in Tissue-Tek OCT compound (Miles), and stored at −80°C. Frozen sections were cut 5–7 μm thick on a cryostat, mounted on Superfrost slides (Fisher Scientific, Pittsburgh, PA), fixed for 20 min in 4% paraformaldehyde at room temperature, and washed in PBS. Endogenous peroxide was blocked with H₂O₂ (3%) in methanol solution for 30 min. Slides were pretreated with 5% normal rabbit serum, 1% BSA, and 0.3% Triton X-100 for 30 min. After a washing in PBS, primary antisera were applied (NK-1R, 1:1,000 to 1:3,000; NK-2R, 1:2,000 to 1:4,000; NK-3R, 1:1,000) in PBS, 1% normal goat serum, and 0.3% Triton X-100 and incubated for 12 h at 4°C in a humidified chamber. Immunoreaction was visualized using the unlabeled antibody-enzyme streptavidin-biotin complex (Vectastain, Vector Laboratories) technique, as previously described (22). After thorough rinsing of slides with PBS, the secondary antibody (1:100 in PBS-0.25% BSA) was applied for 30 min at room temperature. After a washing, the streptavidin-peroxidase complex was added at a dilution of 1:200 for 30 min at room temperature. Finally, staining was visualized by incubation in a solution containing 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.01% H₂O₂ in Tris-buffered saline (pH 7.6). Slides were dehydrated in graded alcohols and xylene and mounted in Permount medium (Fisher Scientific). To control for specificity of immunodetection, antisera were incubated with an excess of the corresponding peptides (10⁻⁵ M) used for immunization and utilized in immunohistochemistry of frozen tissue sections as above (14). As a positive control for each antibody, immunohistochemistry was performed using monolayers of Kirsten murine sarcoma virus-transformed rat kidney cells transfected with the gene for NK-1R or using Chinese hamster ovary (CHO) cells transfected with the NK-2R gene or NK-3R gene (34, 47).

Determination of ICAM-1 mRNA expression by Northern blot analysis. Cultured HDMEC (1 × 10⁶ cells) were treated with 100 nM SP for 1, 3, or 5 h or with 30 μM TNF-α for 5 h. TNF-α is utilized as a positive control for ICAM-1 induction on HDMEC. Total cellular mRNA was isolated, and Northern blot analysis was done as previously described (9). The ICAM-1 probe consists of a 0.67-kb Stu I fragment of the human ICAM-1 cDNA (generously provided by Dr. D. E. Staunton, Dana Farber Cancer Institute) (44). Hybridization with radiolabeled β-actin cDNA was used for determination of lane-loading consistency and for normalization of ICAM-1 signal under the various conditions tested. The autoradiograph was scanned on a La Cie flatbed scanner (La Cie, Beaverton, OR) utilizing Adobe Photoshop software (Adobe Systems, Mountain View, CA). Subsequently, the digitized image was analyzed using NIH Image software (National Institutes of Health, Bethesda, MD), and the relative intensity was calculated as the ratio of pixels per square inch of ICAM-1 to β-actin per lane.

Determination of HDMEC surface expression of ICAM-1 by ELISA. HDMEC were plated onto 96-well plates and on reaching confluence were stimulated with various concentrations of SP for fixed time periods or with a fixed concentration of SP for various time periods. TNF-α at 300 U/ml was used as a positive control for ICAM-1 cell surface expression. For ICAM-1 dose response studies, HDMEC were stimulated with SP at 10, 100, or 1,000 nM for 18 h. For ICAM-1 induction kinetics studies, HDMEC cell surface ICAM-1 expression was assessed by a two-step ELISA as previously described (9), using the mouse anti-human ICAM-1 monoclonal antibody (MAb) 84H10 followed by a peroxidase-conjugated goat anti-mouse IgG that was detected by a 3,3′,5′-tetramethylbenzidine colorimetric re-
Neurokinin receptors are G protein-linked receptors that trigger a rapid increase in intracellular Ca\(^{2+}\) influx and mobilization of intracellular Ca\(^{2+}\) stores (3, 18). The expression of these receptors was demonstrated in Fig. 1, HDMEC express mRNA for neurokinin receptors. SP is capable of specifically binding to NK-1R, NK-2R, and NK-3R with high, intermediate, and low affinity, respectively (3, 18). The expression of these receptors was examined in HDMEC by nonquantitative RT-PCR. As demonstrated in Fig. 1, HDMEC expressed mRNA for NK-1R (487 bp), NK-2R (687 bp), and NK-3R (655 bp). Thus HDMEC express the mRNAs for the three neurokinin receptors capable of binding SP.

RESULTS

SP induces intracellular Ca\(^{2+}\) increase in HDMEC. Neurokinin receptors are G protein-linked receptors that trigger a rapid increase in intracellular Ca\(^{2+}\) when activated (25). To determine whether HDMEC are capable of responding to SP, we examined the ability of SP to induce intracellular Ca\(^{2+}\) influx and mobilization of these cells (Fig. 2). Treatment of HDMEC with 100 nM SP resulted in an immediate and transient increase in intracellular Ca\(^{2+}\) (Fig. 2). The effect of specific antagonists was then examined on SP induction of HDMEC intracellular Ca\(^{2+}\) responses (Fig. 2). As indicated, pretreatment of cells with antagonists for NK-1R, NK-2R, and NK-3R for 20 min before application of treated HDMEC, unlabeled J-Y cells were added according to the same protocol, stopping before the addition of SDS. Photomicrographs were taken using Kodak Ektachrome 66 tungsten film on an Olympus OM-2 camera mounted on an Olympus CK2 microscope.

Determination of in vivo expression of ICAM-1 in human skin microvascular cells by immunohistochemistry. Capsaicin (Zostrix, 0.075%, GenDerm, Lincolnshire, IL) was applied topically to the skin of a volunteer to stimulate the release of cutaneous neuropeptides, and 4-mm punch biopsies were taken from treated sites at 6, 24, and 48 h. Biopsies were also taken from an untreated site on the opposite limb immediately before capsaicin application. Tissue was embedded in OCT and frozen at −70°C. Immunohistochemistry was performed on 8-μm sections using mouse anti-human ICAM-1 MAb 84H10 as the primary antibody (diluted 1:400), a biotinylated horse anti-mouse secondary antibody (Zymed), and streptavidin-biotin complex (Zymed) as a tertiary reagent to detect specific binding of the primary and secondary reagents. Samples were examined using an Olympus BX60 microscope and photographed using a Nikon FX-35 microscope camera. Control staining was performed on tissue processed without the primary antibodies. No staining was obtained with either of the controls.

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Fig. 1. Human dermal microvascular endothelial cells (HDMEC) express mRNA for neurokinin receptors 1, 2, and 3 (NK-1R, NK-2R, and NK-3R). RT-PCR products were amplified from HDMEC mRNA using RT-generated cDNA template and paired primers for neurokinin receptors. PCR amplification products of 487, 687, and 655 bp represent specific fragments of NK-1R, NK-2R, and NK-3R, respectively. Labeled standard lanes indicate that amplification products were of expected sizes. Data are representative of experiments conducted in triplicate.

### RESULTS

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SP completely abrogated the intracellular Ca\(^{2+}\) response. In contrast, pretreatment of cells with the NK-3R antagonist alone had little effect on SP intracellular Ca\(^{2+}\) responses, whereas the NK-2R antagonist was capable of partially blocking this response. Importantly, treatment of cells with NK-1R antagonist alone was capable of completely blocking the SP activation of HDMEC. These data suggest that SP primarily activates HDMEC through NK-1R but that some cross-reactivity of the NK-1R antagonist for NK-2R may exist. Treatment of the cells with Triton X-100 followed by EGTA showed the expected increase in Ca\(^{2+}\) mobilization and subsequent return to baseline. Thus SP is capable of specifically inducing Ca\(^{2+}\) influx and mobilization in HDMEC, an activation signal that could result in the induction of downstream events associated with an inflammatory response. This effect appears to be mediated primarily by the activation of the HDMEC NK-1R.

Microvascular cells express NK-1R in vivo. Neurokinin receptors were immunolocalized in normal human skin using specific antisera to NK-1R, NK-2R, and NK-3R (14). There was significant NK-1R immunostaining of microvascular cells in the dermis (Fig. 3A; see also Fig. 4), but weak immunostaining of these cells was observed with antisera for NK-2R (Fig. 3B). No immunostaining was observed using antisera for NK-3R (data not shown). No immunoreaction was seen when antisera were preabsorbed with an excess of either NK-1R peptide (Fig. 3C) or NK-2R peptide (Fig. 3D), which demonstrates the specificity of the immunostaining. Positive immunostaining was observed using control Kirsten rat kidney cells or CHO cells transfected to express each neurokinin receptor (34, 47) and stained with the respective antisera for that receptor (data not shown). Taken together, these data suggest that the predominant cell surface functional neurokinin receptor on HDMEC in vitro and microvascular endothelial cells in vivo is NK-1R.

SP induces ICAM-1 mRNA expression in HDMEC. To ascertain whether SP is capable of modulating ICAM-1 mRNA in HDMEC, Northern blot analysis was performed (Fig. 4A). HDMEC were treated with 100 nM SP for 1, 3, or 5 h or with 30 U/ml TNF-\(\alpha\). HDMEC ICAM-1 mRNA expression was normalized to \(\beta\)-actin mRNA expression by densitometric analysis for each experimental condition (Fig. 4B). As indicated, HDMEC constitutively expressed low levels of ICAM-1 mRNA, which increased 1.25-fold 1 h after treatment with SP. ICAM-1 mRNA levels further increased 2.5-fold 3 h...
after the addition of SP but decreased to constitutive mRNA expression levels 5 h after the addition of SP. TNF-α induction of HDMEC ICAM-1 mRNA expression served as a positive control for this study. Thus SP is capable of inducing ICAM-1 mRNA expression in HDMEC.

SP induces cell surface ICAM-1 expression on HDMEC. To determine whether increased HDMEC ICAM-1 mRNA expression after SP treatment results in increased ICAM-1 cell surface expression, cells were treated with various concentrations of SP for 18 h, and ICAM-1 expression was measured by ELISA. As indicated in Fig. 5A, SP upregulated ICAM-1 cell surface expression in a concentration-dependent fashion. A fourfold increase in HDMEC ICAM-1 cell surface expression over constitutive levels was observed after treatment with 10 nM SP, and a fivefold increase in ICAM-1 expression was observed after treatment with 100 nM SP (Fig. 5A); 1,000 nM SP was only able to induce a small increase in ICAM-1 expression. We (24) and others (16) have observed dose responses in other neuropeptide studies. Thus 100 nM SP would appear to be the optimal concentration for the induction of cell surface HDMEC ICAM-1.

The kinetics of this SP-ICAM-1 response were also examined. Using a fixed concentration of 100 nM SP, maximum induction of HDMEC ICAM-1 expression was observed between 16 and 18 h after stimulation with SP, inducing a fivefold increase in cell surface ICAM-1 expression compared with untreated HDMEC (Fig. 5B). TNF-α induction of HDMEC ICAM-1 served as a positive control in these studies. These data demonstrate that, in addition to the induction of HDMEC ICAM-1 mRNA, SP upregulates the expression of cell surface ICAM-1 on HDMEC in a concentration- and time-dependent fashion.

The effect of SP on HDMEC ICAM-1 cell surface expression was also examined by flow cytometric analysis. Untreated HDMEC expressed low constitutive levels of surface ICAM-1 (Fig. 6A). As in the ELISA studies, treatment of HDMEC with 10 and 100 nM SP for 18 h resulted in a significant concentration-dependent increase in cells expressing surface ICAM-1 compared with control untreated cells (Fig. 6A, bottom left and top right). TNF-α-treated HDMEC demon-
Fig. 5. ELISA measurement of ICAM-1 expression of SP-treated HDMEC. A: HDMEC were stimulated with 10, 100, and 1,000 nM SP or 300 U/ml TNF-α at 37°C for 18 h, and cell surface ICAM-1 was measured by ELISA. B: HDMEC were also stimulated with 100 nM SP for 1–24 h or with 300 U/ml TNF-α for 18 h at 37°C, and cell surface expression was measured by ELISA. Results are means ± SD of 4 values. *Statistically significant differences in cell surface ICAM-1 in treated samples compared with untreated control cells (–) as determined by Student’s t-test (P < 0.005). Data are representative of experiments conducted in triplicate.

Fig. 6. Flow cytometric analysis of ICAM-1 expression of SP-treated HDMEC. A: surface expression of ICAM-1 on HDMEC was assessed by flow cytometric analysis 18 h after exposure to 10 nM SP (bottom left), 100 nM SP (top right), or 300 U/ml TNF-α (bottom right). B: surface expression of ICAM-1 on HDMEC was also assessed by flow cytometric analysis 18 h after exposure to NK-1RA followed by 100 nM SP (bottom left), 100 nM SP (top right), or 300 U/ml TNF-α (bottom right). Open histogram areas under solid lines represent constitutive ICAM-1 expression (Untx Ctrl); superimposed filled histogram areas represent expression with treatments indicated by heavier solid lines in keys. In A and B, top left shows untreated cells incubated with an isotype control IgG in place of anti-ICAM-1 antibody. Data are representative of experiments conducted in triplicate.
demonstrated high levels of cell surface ICAM-1 expression and served as a positive control (Fig. 6A, bottom right). First-step incubation with an irrelevant isotype-matched control antibody demonstrated the specificity of the ICAM-1-detected signal seen after SP and TNF-α (Fig. 6A, top left).

As indicated in Fig. 6B, pretreatment of HDMEC with an NK-1R antagonist followed by the addition of 100 nM SP blocked the SP induction of cell surface ICAM-1 nearly to the level of constitutive ICAM-1 expression of untreated cells (Fig. 6B, bottom left). Treatment of HDMEC with 100 nM SP alone resulted in a significant increase in cells expressing surface ICAM-1 (Fig. 6B, bottom left and top right). TNF-α-treated HDMEC again served as a positive control (Fig. 6B, bottom right), and first-step incubation with an irrelevant isotype-matched control antibody demonstrated the specificity of the ICAM-1-detected signal (Fig. 6B, top left).

SP enhances binding of J-Y lymphoblastoid cells to HDMEC. The functional consequences of SP induction of cell surface ICAM-1 on binding of J-Y lymphoblastoid cells to HDMEC were determined using a quantitative cellular adhesion assay (10). Although 51Cr-labeled J-Y cells showed minimal binding to untreated cultured HDMEC, the adhesion of J-Y cells to SP-treated HDMEC increased in a concentration-dependent manner following the addition of 10 or 100 nM SP for 18 h (Fig. 7). As indicated, 100 nM SP induced a threefold increase in adhesion of J-Y cells to HDMEC. There was little or no increase in J-Y cell adhesion to HDMEC treated with 1,000 nM SP, reflecting our previous studies that demonstrated a poor ICAM-1 cell surface inductive response after this high concentration of SP (data not shown). Contribution of ICAM-1 to the adherence of J-Y cells to HDMEC after SP treatment was examined. Leukocyte binding to SP-treated HDMEC could be prevented by pretreatment of HDMEC with an anti-ICAM-1 blocking antibody before the addition of labeled J-Y cells (Fig. 7). The anti-ICAM-1 antibody alone, or an anti-VCAM-1 antibody used as an irrelevant antibody (data not shown), had little effect on J-Y cell binding to HDMEC. TNF-α treatment of HDMEC served as a positive control for induction of J-Y binding.

The ability of SP to augment J-Y cell binding to HDMEC was also visually demonstrated in photomicrographs of the cellular adhesion assay (Fig. 8). Untreated HDMEC showed minimal numbers of adherent J-Y cells (Fig. 8A), whereas HDMEC treated with 100 nM SP showed high numbers of adherent J-Y cells (Fig. 8B). Again, adhesion of J-Y cells to HDMEC could be blocked by the addition of the anti-ICAM-1 antibody to the SP-treated cells (Fig. 8C). As expected, large numbers of J-Y cells adhered to HDMEC monolayers pretreated with TNF-α (Fig. 8D). These data demonstrate that SP is capable of augmenting ICAM-1-dependent binding of LFA-1-expressing lymphoblastoid cells to HDMEC.

In vivo ICAM-1 induction in capsaicin-treated human skin. The effect of in vivo release of cutaneous neuropeptides on dermal endothelial cell ICAM-1 expression was examined. A capsaicin-containing cream (Zostrix) was applied to the skin of a human volunteer to stimulate the release of neuropeptides, including SP from cutaneous sensory nerves (31). Biopsies were obtained at 0, 6, 24, and 48 h and were evaluated by immunohistochemistry for ICAM-1 expression associated with HDMEC. Low constitutive levels of microvascular ICAM-1 immunoreactivity were observed in untreated skin (Fig. 9a), and endothelial cell ICAM-1 expression gradually increased at 6 h post-capsaicin application (Fig. 9b). A marked increase in HDMEC ICAM-1 staining was observed by 24 h after application of topical capsaicin (Fig. 9c), and the increase remained 48 h after capsaicin application (Fig. 9d). Thus the release of neuropeptides by cutaneous sensory nerves results in increased in vivo microvascular endothelial ICAM-1 expression.

**DISCUSSION**

There is increasing evidence that skin inflammation can be mediated by the cutaneous neurological system through the release of neuropeptides, such as SP, that interact with target skin cells. The close physical association of cutaneous nerves with target cells has been established (21, 49, 50). SP has been well characterized as a potent vasodilator that can cause increased microvascular permeability and protein extravasation (11). Additionally, SP can induce leukocyte effector activities, such as lymphocyte proliferation, cytotoxicity, and immunoglobulin production (3, 36); mast cell...
degranulation (37); macrophage and polymorphonuclear leukocyte activation (17, 51); and cytokine production by monocytes (23, 28, 30). Our previous studies indicate that SP can directly activate mast cells and keratinocytes to secrete TNF-α and interleukin-1 (IL-1), respectively (2, 4).

The assessment of cutaneous neuropeptide modulation of HDMEC adhesion molecule expression is important to our understanding of interactions between the cutaneous neurological system and skin inflammatory responses. Inflammatory infiltration of leukocytes into all tissues, including the skin, depends on leukocyte passage into tissue from the microvasculature in a multi-step binding interaction involving cell adhesion molecules expressed by leukocytes and endothelial cells (27, 48). P- and E-selectins mediate initial leukocyte adhesion to HDMEC through binding to specific carbohydrate moieties present on neutrophils, whereas ICAM-1 and VCAM-1 mediate subsequent, firm adhesion and transendothelial migration through binding to β2 and β1-integrins on leukocytes. In the skin, HDMEC expression of ICAM-1 is an essential component of cutaneous inflammation. We have previously shown that ICAM-1 is constitutively expressed in vitro by HDMEC and that the proinflammatory cytokines interferon-γ, TNF-α, and IL-1, as well as ultraviolet B radiation, increase ICAM-1 surface expression (7, 45). The direct effect of neuropeptides on HDMEC ICAM-1 expression has not been previously examined.

Our present evaluation of the effect of SP on cutaneous microvascular endothelial cell ICAM-1 expression significantly expands our understanding of the role of the cutaneous neurological system in inflammatory responses in the skin. Our results indicate that cultured HDMEC express mRNA for NK-1R, NK-2R, and NK-3R, which are capable of binding to SP with high, intermediate, and low affinity, respectively. We demonstrate that SP induces a rapid intracellular Ca2+ re-
response in HDMEC and that this effect is mediated primarily by NK-1R. In vivo, immunohistochemistry studies show that NK-1R is the major neurokinin receptor expressed on dermal microvascular cells. HDMEC activation is further accompanied by increased levels of ICAM-1 mRNA and ICAM-1 cell surface expression. In parallel, increased leukocyte binding is observed in SP-treated HDMEC. A similar induction of HDMEC ICAM-1 expression is observed in vivo after the topical application of the SP-releasing agent capsaicin.

Our investigation of the role of microvascular endothelial ICAM-1 upregulation in neurogenic inflammation is supported by several prior studies that suggest neuropeptides may be capable of influencing adhesion molecule expression on these target cells in the skin. We believe that our study is the first to definitively demonstrate that SP is capable of directly activating HDMEC to express increased levels of functional ICAM-1 in vitro and in vivo. In a previous study, Nakagawa et al. (33) reported that SP can upregulate ICAM-1 expression in vitro on large-vessel human umbilical vein endothelial cells. However, this study did not evaluate the presence and function of neurokinin receptors on these cells or the functional consequences of ICAM-1 upregulation, nor was there in vivo correlation examined. Because all tissue inflammatory responses in the skin and elsewhere are mediated by microvascular endothelial cell activities rather than large-vessel endothelial cells and because the two cell types display distinct differences in their phenotypes and responses to proinflammatory signals (46), we believe that it is critical to examine HDMEC when studying neuroinflammation responses in the skin.

Some prior studies have utilized murine models to begin to evaluate adhesion molecule expression following administration of neuropeptides. Saban et al. (41) focused on the responses of neutrophils and eosinophils to SP following injection in mouse skin. ICAM-1 involvement was indirectly demonstrated in terms of the ability of pretreatment of mice with MAb to ICAM-1 to inhibit SP-induced leukocyte migration, but there was no determination of ICAM-1 expression on endothelial cells. Goebeler et al. (13) examined the effect of SP and another neuropeptide, calcitonin gene-related peptide (CGRP), on leukocyte infiltration during allergic contact dermatitis in mouse skin. These authors measured increased ICAM-1 on tissue macrophages and did not observe an upregulation of ICAM-1 on SP-treated murine endothelial cell lines in vivo.

Using an in vivo human skin model system, Smith et al. (43) demonstrated that injection of the neuropeptides SP, vasoactive intestinal polypeptide, and CGRP into normal human skin mediated a rapid accumulation of neutrophils. Using immunohistochemistry, these investigators noted a parallel upregulation of P-selectin and E-selectin over an 8-h time period, but constitutive ICAM-1 expression on dermal endothelium was unchanged at these early time points after neuropeptide injection. These findings are consistent both with the expected time course of ICAM-1 upregulation (6, 7) and with the results of our current study, which indicate that microvascular ICAM-1 immunoreactivity was significantly increased over constitutive levels at 24 and 48 h after treatment with capsaicin. Murphy and colleagues (29) have demonstrated that SP treatment of skin explants caused mast-cell degranulation and subsequent TNF-α-mediated induction of E-selectin on postcapillary venular endothelial cells. In contrast to these findings, we observed that SP was capable of directly inducing increased HDMEC ICAM-1 expression without the participation of mast cells. In vivo, both processes are likely to play a role.

In summary, our studies demonstrate that SP is capable of directly regulating HDMEC ICAM-1 expression and function by the activation of specific neurokinin receptors. 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 upregulation may play a role in the development of a wide range of inflammatory responses and during wound healing. We also have recent evidence that SP is capable of specifically inducing another adhesion molecule, VCAM-1, on HDMEC, which further suggests that this neuropeptide may be involved in multiple inflammatory responses in the skin (1). Further investigation of the interactions of various neuropeptides, adhesion molecules, and other inflammatory mediators in the skin should provide a rational basis for formulating an effective, specific approach to the control of neurogenic inflammation. Such approaches could lead to novel therapies that have application to a wide range of inflammatory processes, such as psoriasis, atopic eczema, and wound healing.

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Address for reprint requests: J. C. Ansel, Dept. of Dermatology, Emory University School of Medicine, 5001 Woodruff Memorial Bldg., Atlanta, GA 30322.

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