Substance P Differentially Stimulates IL-8 Synthesis in Human Corneal Epithelial Cells

Mau T. Tran, Robert N. Lausch, and John E. Oakes

OBJECTIVE. To determine whether substance P (SP), a neuropeptide with proinflammatory properties, specifically interacts with human corneal epithelial cells to stimulate synthesis of the chemokines interleukin (IL)-8, monocyte chemo-attractant protein (MCP)-1, and regulated on activation normal T-cell expressed and secreted (RANTES) protein.

METHODS. Primary cultures of human corneal epithelial cells were established from human corneas. Expression of the SP receptor neurokinin (NK)-1 was determined by both the reverse transcription-polymerase chain reaction (RT-PCR) and radiolabeled saturation binding experiments. Synthesis of chemokine-specific RNA in cells stimulated with SP was analyzed by RT-PCR, and quantitation of chemokine protein synthesis was achieved by enzyme-linked immunosorbent assay.

RESULTS. Human corneal epithelial cells expressed NK-1 mRNA and bound SP with a K_d characteristic of NK-1. Exposure of cells to SP had no effect on IL-8-specific mRNA synthesis, whereas it increased the half-life of IL-8 transcripts by more than twofold, resulting in significant enhancement of IL-8 synthesis. The capacity of SP to bind to corneal epithelial cells and to induce IL-8 synthesis was abrogated in the presence of a specific NK-1 receptor antagonist. In contrast to IL-8, exposure of cells to SP did not stimulate synthesis of MCP-1 or RANTES.

CONCLUSIONS. The results suggest that human corneal cells express NK-1 receptors that specifically bind SP and induce IL-8 synthesis by stabilizing the chemokine’s transcripts. (Invest Ophthalmol Vis Sci. 2000;41:3871–3877)

Substance P [SP] is a low-molecular-weight neuropeptide stored in afferent and efferent termini of sensory neurons.1 Within the central nervous system, release of the neuropeptide from efferent sensory nerve termini contributes to transmission of pain sensations.2-5 Outside the central nervous system, depolarized sensory neurons release SP from afferent terminals directly into peripheral tissues where the neuropeptide can initiate numerous inflammatory reactions including vasodilation of microvascular blood vessels,4 stimulation of leukocyte adhesion molecule expression,5,6 chemoattraction and activation of neutrophils,7,8 and induction of mast cell degranulation, histamine release, and cytokine synthesis.9,10 When released into lymphoid tissue, SP can regulate T- and B-cell activities,11-15 and induce monocytes to synthesize numerous regulatory cytokines including interleukin (IL)-1, IL-6, IL-10, IL-12, and tumor necrosis factor (TNF)-α.14-16 SP may also be involved in inducing inflammatory responses within the brain by stimulating IL-6 and IL-8 synthesis in astrocytes.17,18

Neutrophils play an important role in protecting corneal tissues from pathogens and foreign substances that impinge on the eye surface.19,20 However, the cornea has no blood supply or lymphatic drainage. Thus, the nearest source of neutrophils is found in limbic blood vessels surrounding clear cornea surfaces. IL-8, a member of the chemokine family of proinflammatory mediators, is noted for chemoattraction of neutrophils into inflamed tissues.21 Because human corneal epithelial cells selectively synthesize IL-8 in response to proinflammatory mediators, this chemokine is assumed to play an important role in the chemoattraction of neutrophils to sites of acute inflammation at corneal surfaces.22,23

SP mediates its effects by binding to specific SP receptors expressed by peripheral and central nervous system tissues.1 These receptors include the high-affinity SP-binding receptor neurokinin (NK)-1 and low-affinity SP-binding receptors NK-2 and NK-3.24 It has recently been discovered that calcitonin gene-related peptide (CGRP), a neuropeptide found in sensory afferent nerve termini innervating the epithelial layer of the cornea, binds to CGRP receptors on human corneal epithelial cells to induce IL-8 synthesis.25 With the knowledge that SP is released into epithelial layers of the cornea at the same time as CGRP,26,27 we initiated this study to determine whether SP can also act as an IL-8 inducer within the epithelium of the human cornea. SP readily induced IL-8 synthesis in human corneal epithelial cells by interacting with surface NK-1 receptors to initiate a signal transduction pathway that led to enhanced stability of IL-8 transcripts. The significance of these findings in the development of acute inflammation within the eye surface is discussed.

From the 1Laboratory of Experimental Carcinogenesis, National Cancer Institute, National Institutes of Health, Bethesda, Maryland; and the 2Department of Microbiology and Immunology, College of Medicine, University of South Alabama, Mobile.

Supported by Grant EY12713 from the National Institutes of Health and the Lions USA Eye Research Foundation.

Submitted for publication May 16, 2000; revised July 14, 2000; accepted July 21, 2000.

Commercial relationships policy: N.

Corresponding author: John E. Oakes, Department of Microbiology and Immunology, MSB 2096, College of Medicine, University of South Alabama, Mobile, AL 36688. joakes@jaguar1.usouthal.edu
MATERIALS AND METHODS

Preparation of Corneal Epithelial Cells

Human corneas were obtained from the National Disease Research Interchange (Philadelphia, PA) and processed within 48 hours of enucleation, as described previously. It has been shown that cultivation of epithelial cells by this technique results in the establishment of pure cultures. Cultures were grown to confluence in 25-cm² flasks using keratinocyte serum-free medium (Gibco, Grand Island, NY) as the growth medium. The cell cultures were then stimulated with the desired concentration of SP and at selected times after stimulation, the supernatants were removed and stored at -20°C for subsequent cytokine assays.

Cytokine and Cyclic Adenosine Monophosphate Assays

Synthetic SP and an NK-1 receptor agonist (Spanptide; purity of >97%) were purchased from Sigma (St. Louis, MO). Human IL-8, monocyte chemo-attractant protein (MCP-1), and regulated on activation normal T-cell expressed and secreted (RANTES) protein levels were quantified by using enzyme-linked immunosorbent assay (ELISA) kits (R&D; Minneapolis, MN) with a detection limit of 3.0 pg/ml for IL-8 and 5 pg/ml for MCP-1 and RANTES. Colorimetric results were read at 450 nm by microplate reader (EL308; Biotek Instruments, Winooksi, VT). Significance differences in chemokine synthesis were determined by using small-sample paired t statistics. P < 0.05 was considered significant.

SP Receptor Binding Assay

Cultures of epithelial cells were grown to confluence in 48-well plates. The cells were then washed twice with ice-cold binding buffer (140 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 0.9 mM MgCl₂, 25 mM HEPES [pH 7.4], 1 mM 1,10-phenanthroline, 1 mM glucose, and 1% bovine serum albumen [BSA] in phosphate-buffered saline [PBS]) and exposed to from 1 × 10⁻³ to 1 × 10⁻¹⁰ M of SP. The cells were then lysed using 200 μl PBS to remove all unbound ligand and combined with the tubes containing cell extracts. The lysates and supernatant aliquots were counted separately for radioactivity in a scintillation counter using a scintillation cocktail (Lexilux; Beckman, Fullerton, CA) to determine bound and unbound ligand with a count efficiency of more than 90%. Scatchard analysis was performed by computer (Prism program; GraphPad, San Diego, CA). Nonspecific binding was found to be less than 15%.

Total RNA Isolation

Epithelial cell cultures were established from individual corneal donors. After treatment with SP, supernatants were removed from cultures and total RNA isolated by using the acid guanidine thiocyanate-phenol-chloroform extraction method. RNA was fractionated on a 1.0% agarose gel containing 2.2 M formaldehyde and then stained with 1 mg/ml ethidium bromide to confirm that RNA spectrophotometric measurements were accurate and that the RNA had not been degraded.

Primer Selection

Polymerase chain reaction (PCR) primers were selected with the aid of a computer program (OLIGO primer selection software; Eccles Institute for Human Genetics and Howard Hughes Medical Institute, University of Utah, Salt Lake City) run on the National Cancer Institute–Frederick Cancer Research Center’s Vax 6620 (Frederick, MD). The primers were complementary to mRNA sequences within the human IL-8, growth-related oncogene (GRO)-α, MCP-1, RANTES, and glyceraldehyde phosphate-dehydrogenase (GAPD) coding regions. The primers for mRNA amplification spanned at least one intron. The primers selected for each chemokine mRNA amplification were as follows: human (h)NK-1 mRNA (231 bp): sense 5'-TAT GAG GGG CTG GAA ATG AAA TC-3' and antisense 5'-TAG GAG AGC ACA TTG GAG GAA ATG AAA TC-3'; and hIL-8 mRNA (218 bp): sense 5'-CTC TCT TGG GAG CCT CTC TGA TT-3' and antisense 5'-AAC TTT TCC ACA ACC CAC CTG AGC-5'.

Primers selected to amplify pre-mRNA for IL-8 were chosen so that the forward primer was placed within an intron sequence, and the reverse primer was placed within an exon sequence. Because there was a possibility of picking up genomic DNA, a set of reverse transcription–PCR (RT-PCR) control samples were run in parallel throughout the RT-PCR reactions that contained no reverse transcriptase or RNA to rule out genomic DNA amplification. Primers used for GAPD amplification have been described. Primers used for amplification of IL-8 pre-mRNA were: hIL-8 pre-mRNA (592 bp): sense 5'-TAT GAT GCC TTC CAT AGT CTC CA-3' and antisense 5'-AAC TTT TCC ACA ACC CAC CTG AGC-5'.

Amplified PCR products were sequenced at The Biopolymer Center (Mobile, AL) on an automatic sequencer (model 373XL; Perkin-Elmer/Applied Biosystems, Foster City, CA).

Analysis of IL-8 mRNAs and Pre-mRNA Levels by RT-PCR

cDNA strands complementary to total cellular RNA were made by using a kit (GeneAmp; Perkin-Elmer), adding 1 mg total cellular RNA, random hexamer, and 2.5 U/μl M-MLV reverse transcriptase in a 20-μl total volume. In mRNA stability assays, 10 μg/ml actinomycin D was added to cell cultures 1 hour after stimulation with 1 μM SP. At selected times after actinomycin D treatment total RNA was harvested, and specific RNA molecules amplified by RT-PCR. All RT-PCR products were amplified using thermocycles of 30 seconds at 95°C, 30 seconds at 65°C, and 2 minutes at 72°C. Duplicate samples without RNA or without RT were amplified by PCR to verify that the RNA samples did not contain detectable levels of genomic DNA. Each PCR sample was then analyzed on a 1.5% agarose gel stained with 1 μg/ml ethidium bromide and viewed and photographed (Digital Science SP700 camera; Kodak Scientific Imaging Systems, New Haven, CT). The digitized negatives of the PCR products were quantitated using the accompanying software (Kodak Scientific Imaging Systems).
RESULTS

Effect of SP on IL-8 Synthesis

Preliminary experiments were performed to determine whether SP induces IL-8 synthesis in human corneal epithelial cells. Confluent monolayers of human corneal epithelial cells were stimulated with various concentrations of SP. At 2 hours after stimulation, supernatants were collected and assayed for IL-8. As noted in earlier studies, unstimulated human corneal epithelial cells produced small constitutive levels of IL-8 (Fig. 1, inset). However, cells exposed to doses of SP ranging from 0.01 to 1 \( \mu \)M synthesized significantly greater levels of IL-8 than unstimulated cells (\( P < 0.05 \)). It can be estimated from these experiments that the dosage of SP that elicits a half-maximal response (ED\(_{50}\)) is approximately 0.005 \( \mu \)M (5.0 \( \times \) 10\(^{-9} \) M), which is close to the ED\(_{50}\) reported for SP stimulation of phosphatidylinositol hydrolysis and cyclic adenosine monophosphate (cAMP) responses. Spantide is a specific NK-1 receptor antagonist. The dependence of IL-8 gene expression on interactions between SP and NK-1 receptors was confirmed by demonstrating that IL-8 synthesis was not induced in cultures exposed to SP in the presence of the NK-1 receptor agonist (Fig. 3). Thus, specific inhibition of the binding of SP to NK-1 receptors blocks induction of IL-8 synthesis.

Detection of NK-1 Receptors on Human Corneal Epithelial Cells

NK-1 molecules are the principle SP receptors expressed on peripheral tissues. Therefore, we used RT-PCR to determine whether the gene for the NK-1 receptor is transcribed by human corneal epithelial cells. It was found that an RT-PCR product complementary to NK-1 receptor mRNA could be amplified easily from purified human corneal epithelial cell RNA (data not shown). To confirm that NK-1 receptor transcripts were expressed as cell surface SP-binding proteins, equilibrium-binding experiments with \(^{125}\)I-SP were performed. Analysis of the steady state binding of \(^{125}\)I-SP to human corneal epithelial cells indicated that SP binds to these cells in a specific and saturable manner (Fig. 2). Scatchard analysis of the binding data indicated that human corneal epithelial cells have approximately 820 binding sites per cell with a \( K_d \) value of 3.6 \( \times \) 10\(^{-9} \) M (Fig. 2, inset). The \( K_d \) is consistent with \( K_d \) values previously reported for human SP receptors.

Effect of SP on IL-8 Transcripts

One mechanism whereby proinflammatory stimuli could regulate IL-8 synthesis in corneal epithelial cells is by enhancing transcription of the IL-8 gene. To determine whether SP enhances IL-8 synthesis by upregulating production of IL-8-specific transcripts, we monitored IL-8 pre-mRNA synthesis after exposure of human corneal epithelial cells to SP. It was found that IL-8 pre-mRNA synthesis was not significantly enhanced in SP stimulated cells (Fig. 4). However, when IL-8 mRNA levels were analyzed by RT-PCR, it was found that SP stimulation increased steady state levels of IL-8 mRNA by almost fourfold (Fig. 4). These results suggest that SP may regulate IL-8 synthesis at the level of mRNA stability rather than at the level of RNA synthesis. To explore this possibility, human corneal epithelial cells were stimulated with SP for 1 hour and...
then treated with actinomycin D to inhibit transcription of the IL-8 gene. Steady state levels of IL-8 mRNA were then analyzed by RT-PCR. It was found that the half-life of IL-8 mRNA in unstimulated cultures was less than 1.5 hours (Fig. 5A). In contrast, the half-life of IL-8 mRNA in SP-stimulated cultures was more than two times longer (3.5 hours; Fig. 5B). It was evident that actinomycin D inhibited transcription of the IL-8 gene in these experiments, because the synthesis of IL-8 pre-mRNA was no longer detected 1 hour after actinomycin D treatment (Fig. 5). These results suggest that SP enhances IL-8 synthesis in human corneal epithelial cells by increasing the stability of the chemokine’s transcripts.

**Effect of SP on MCP-1 and RANTES Gene Expression**

It has been shown that exposure of human corneal epithelial cells to chemokine inducers does not enhance synthesis of MCP-1 or RANTES. Therefore, it was of interest to determine whether SP could stimulate human corneal epithelial cells to produce these two β-chemokines. To test this possibility, medium was removed from SP-stimulated cultures and assayed for MCP-1 and RANTES (Fig. 6). It was found SP did not stimulate significant increases in either MCP-1 or RANTES production. In addition, SP stimulation did not induce detectable increases in steady state levels of either RANTES or MCP-1 transcripts (data not shown). It was evident that the cells were metabolically active in these experiments, because SP induced synthesis of significant levels of IL-8. These results suggest therefore that the genes for RANTES and MCP-1 are not upregulated in human corneal epithelial cells in response to SP.

**DISCUSSION**

The human cornea is densely innervated with SP-containing sensory nerve termini. NK-1 receptors specific for this neuropeptide have been identified on rabbit corneal epithelial cells, where they are believed to play a role in wound healing.
Figure 5. Stability of IL-8 mRNA in corneal epithelial cells after stimulation with SP. Confluent monolayers of human corneal cells were stimulated with 1.0 μM SP or medium alone. One hour after stimulation, 10 μg/ml of actinomycin D was added to the cell cultures. After actinomycin D treatment, total RNA was isolated, and the levels of IL-8 pre-mRNA and mRNA analyzed by RT-PCR. GAPD mRNA was also amplified by RT-PCR to correct for any variation in RNA content between samples and to verify equal RNA loading. The IL-8 mRNA-specific RT-PCR products were normalized to the relative densities of the GAPD mRNA products and plotted. Arrows: Times in which approximately one half of the IL-8 transcripts remained after treatment with actinomycin D. (A) IL-8 mRNA stability in unstimulated cells after actinomycin D treatment. (B) IL-8 mRNA stability in SP-stimulated cells after actinomycin D treatment. Identical experiments were performed using two additional donors, with similar results.

Figure 6. Effects of SP on production of MCP-1 and RANTES. Human corneal epithelial cells were stimulated with 1.0 μM SP. At selected times after stimulation, culture media were assayed for IL-8, MCP-1, and RANTES by ELISA. The results represent the mean ± SEM from three different donors (*P < 0.05).
diseased or damaged corneal tissue. The capacity of SP to stimulate IL-8 synthesis in corneal epithelial cells at the post-transcriptional level may help initiate acute inflammation within the corneal epithelium after injury or infection and may provide a mechanism for amplifying proinflammatory signals sent to epithelial cells by other chemokine inducers. It has recently been found that certain viral pathogens can infect corneal epithelial cells without enhancing release of chemokine inducers such as IL-1α. Neuropeptides released from sensory neurons in response to pain sensations caused by virus replication on corneal surfaces may be another mechanism whereby chemokines such as IL-8 are induced.

References

40. Tran, MT, Tellaex–Isusi M, Elner V, Strieter RM, Lausch RN, Oakes JE. Proinflammatory cytokines induce RANTES and MCP-1 synthesis in human corneal keratocytes but not in corneal epith-


47. Wodnar-Filipowicz A, Moroni C. Regulation of interleukin 3 mRNA expression in mast cells occurs at the posttranscriptional level and is mediated by calcium ions. Proc Natl Acad Sci USA. 1990;87:777–781.


53. Tran MT, Dean DA, Lausch RN, Oakes JE. Membranes of herpes simplex virus type-1-infected human corneal epithelial cells are not permeabilized to macromolecules and therefore do not release IL-1α. Virology. 1998;244:74–78.