ELSEVIER

Endogenous Substance P in Corneal Epithelial Cells and Keratocytes

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Purpose: To detect endogenous substance P (SP) and neurokinin receptor 1 (NK1R) in cultured human corneal epithelial cells (HE) and cultured human keratocytes (HK).

Methods: Messenger RNA (mRNA) expression of SP and endogenous SP in HE and HK was investigated by reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay. mRNA expression of NK1R in HE and HK was investigated by RT-PCR.

Results: The mRNA expression of SP and endogenous SP were recognized in HE and HK. Furthermore, the mRNA of NK1R was expressed in HE and HK.

Conclusion: It was suggested that endogenous SP regulates the biological functions of HE and HK in autocrine or paracrine fashion. **Jpn J Ophthalmol 2002;46:616–620** © 2002 Japanese Ophthalmological Society

Key Words: Enzyme-linked immunosorbent assay, epithelial cells, keratocytes, reverse transcription-polymerase chain reaction, substance P.

Introduction

In the cornea, the trigeminal nerve terminal that secretes substance P (SP) is distributed in the corneal epithelium.[1] In the rabbit cornea, SP is reported to stimulate the proliferating activity of the corneal epithelium in vitro and in vivo.[2–5] The shortage of SP associated with trigeminal paralysis is mentioned as one of the causes of neuroparalytic keratitis.[1–4]

Regarding neurokinin 1 receptor (NK1R), a receptor of SP, Nishida et al[2] have reported that NK1R is concerned with SP's effect in the elongation of the tissue cultured rabbit corneal epithelium in the presence of insulin-like growth factor-1. Araki et al[6] have confirmed the expression of the messenger RNA (mRNA) of NK1R in the human corneal epithelium transformed by SV40 adenovirus.

In recent years, it has been pointed out that SP is produced in cells other than nerve tissue and has a variety of physiological effects.[7–11] To our knowledge, however, there has been no report on whether cells constituting the cornea produce SP on their own. For the purpose of examining the mechanism of SP's action in the human cornea, we investigated the possibility that cultured human corneal epithelial cells (HE) and keratocytes (HK) produce SP and whether NK1 receptor (NK1R), an SP receptor, exists in these cells. This paper is a report of our findings.

Materials and Methods

Cell Culture

The corneas from autopsied eyes of 2 female and 1 male patients in their 70s were used after obtaining

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consent from their bereaved families. On pathological examination of the globes, no disease except cataract was noted. Epithelial cells were established in accordance with the method of Allman et al.[12] The peripheral region of the cornea from the autopsied eyes was cut out and divided into eight equal parts. Each corneal section was placed on a 60-mm culture dish or a 24-well plate for culture in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (DMEM + 10% FCS). After a 3-day culture, corneal epithelial cells had grown around the corneal section, which was removed to obtain HE in primary culture. These primary culture HE cells were grown to semi-confluence, and then used in the experiment.

The keratocytes were established according to the method of Nakayasu et al.[13] The epithelium was removed from the cornea of the autopsied eye with a razor blade and cut into eight corneal sections, the same as the HE. Each section was placed in a 60-mm culture dish with DMEM + 10% FCS, and the keratocytes were cultured around the corneal section for 2 weeks. HK of the third to fifth passage were used in the experiment.

Fibroblasts derived from cultured human skin (HF) were established by the method of Kiss et al.[14] After obtaining the consent of the surgically treated patients and their kin, skin sections of 10 × 10 mm were placed on a 35-mm dish and cultured in DMEM + 10% FCS. After 2 weeks, fibroblasts had proliferated around the skin sections. The third passage HF were used in the experiment.[15]

RNA Extraction From Cells and cDNA Synthesis

RNA extraction from cells was done by acid guanidinium thiocyanate-phenol-chloroform extraction in accordance with the method of Ho et al.[11] HE, HK, and HF cells were cultured to the semi-confluent level in DMEM + 10% FCS, and RNA was extracted by adding an extractant (Trizol; Invitrogen, Faraday, CA, USA) thereto. After extraction of RNA, the cells were centrifuged at 12,000g for 15 minutes, and isopropanol was used for precipitation of RNA. After washing the precipitated RNA with 75% ethanol, 50 μL of diethyl pyrocarbonate treated water (DEPC) was added for the dissolution of RNA.

A reverse transcription-polymerase chain reaction (RT-PCR) kit (Superscript Preamplification System; Invitrogen) was used for cDNA synthesis. For synthetic reaction, DEPC was added to RNA (2 μg) and oligo dT 1 μL (0.5 μg) to make 12 μL of sample/oligo (dT) primer mixed solution, and incubation was done at 70°C for 10 minutes. Additionally, 10× PCR buffer 2 μL, 25 mM $MgCl_2$ 2 μL, 10 mM dNTP mix 1 μL, and 0.1 M DTT 2 μL were added. After incubation of the mixture at 42°C for 5 minutes, inverse transcription enzyme (Superscript II; Invitrogen) 1 μL (200 U) was added, and the mixture was incubated at 42°C for 50 minutes to synthesize cDNA.

mRNA Examination of SP

RT-PCR was performed on the cDNA of HE and HK cells. The PCR reaction mixture contained cDNA 5 μL (0.1 mM), dNTP mix 5μL, 10× PCR buffer 5μL, 10 nM primer 2.5 μL, Taq polymerase 0.25 μL (1.25 U), and H_2O for a total of 50μL.

As specific SP primers, 5′-CGACAGCGACCAGATCAAGGAGG-3′(sense) and 5′-TGCATTGCACTCCTTTCAT-3 (anti-sense), confirmed by Ho et al,[11] were used. The reaction conditions for RT-PCR were as follows: 45 cycles, thermal degeneration at 94°C for 1 minute, annealing at 60°C for 1 minutes and elongation reaction at 72°C for 1 minute. PCR products were subjected to electrophoresis with 3% agarose gel, and analyzed by ethidium bromide staining. A genetic amplifier (PTC-150, Minicycler; MJ Research, Waltham, MA, USA) was used for PCR reaction.

ELISA of SP

ELISA of the SP produced by HE and HK cells at the protein level was done with a commercially available kit (Substance P Enzyme Immunoassay Kit; Cayman, Ann Arbor, MI, USA) in accordance with the method of Ho et al.[11] The measurement was taken three times. After having incubated HE and HK cells up to semi-confluence on a 24-well plate, the culture medium was replaced with serum-free DMEM, and culture was continued for 24 hours. After 24 hours, the supernatant was collected, and 50 μL thereof, together with a rabbit anti-SP antibody and acetylchloline esterase molecule, a labeled antigen conjugating with the rabbit anti-SP antibody in competition with SP (SP tracer), was made to react on a 96-well microplate at 4°C for 24 hours. After 24 hours, the unchanged supernatant and antibodies were washed, and a coloring substrate was added. The mixture was made to react, shielded from light, at room temperature for 2 hours. After reaction, absorbance was read at 420 nm with a microplate reader (Model 450; Bio-Rad, Richmond, CA, USA) and the SP tracer conjugated with the rabbit anti-SP antibody was measured. From the rabbit anti-SP an-

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tibody that was added in a fixed amount and SP tracer, the amount of SP conjugated with the rabbit anti-SP antibody in competition with the SP tracer in the supernatant was calculated.

mRNA Examination of NK1R and G3PDH

After preparing the PCR reaction mixture as described above, RT-PCR was performed under these reaction conditions: 50 cycles, thermal degeneration at 94°C for 1 minute, annealing at 50°C for 1 minute and elongation reaction at 72°C for 1 minute. Specific primers of NK1R, 5′-GGTGATTGGCTATGCATACACC-3′(sense) and 5′TGACGGAACCTG TCATTGAGG-3′ (anti sense), confirmed by Hiramoto et al,[16] were used. RT-PCR was performed on the cDNA of HE, HK, and HF cells.

For each type of cell, PCR was done with G3PDH as the control. The primers used were 5′ACCACA GTCCATGCCATCAC-3 (sense) and 5′-TCCACCACCCTGTTGCTGTA-3′ (anti-sense). Conditions for reaction were 25 cycles, thermal degeneration at 94°C for 1 minute, annealing at 60°C for 1 minute and elongation reaction at 72°C for 1 minute. The same genetic amplifier described above was used. The PCR products were subjected to electrophoresis with 1% agarose gel and analyzed as described previously.

Results

mRNA Expression of SP

Using specific primers to examine the mRNA expression of SP, RT-PCR was performed. As a result, a DNA band was confirmed that corresponds to 219 base pairs (bp) in HE, and 219 bp and 264 bp in HK among the sizes for SP: 210 bp, 219 bp, and 264 bp (Figure 1).

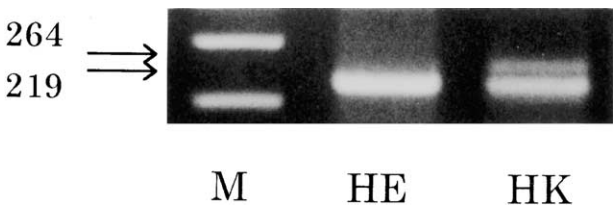

Figure 1. Messenger RNA (mRNA) expression of substance P (SP) in cultured human corneal epithelial cells (HE) and keratocytes (HK) by reverse transcription-polymerase chain reaction (RT-PCR) analysis. mRNA of SP was expressed in HE and HK by RT-PCR analysis using specific primers. The expressed sizes of respective RT-PCR products amplified by using the primers were HE: 219 bp, HK: 219 and 264 bp. M: DNA marker.

Assay of SP Produced by HE and HK

Absorbance was read at 420 nm, and the SP protein amount was measured. The SP amount in the supernatant was found to be 9.9 ± 0.9 pg/mL in HE and 6.4 ± 2.6 pg/mL in HK (Figure 2).

mRNA Expression of NK1R and G3PDH

Using specific primers to examine the mRNA of NK1R, RT-PCR was performed. As a result, a DNA band corresponding to 312 bp was confirmed in both HE and HK (Figure 3A).

Using HF as the positive control, a band equal to that in HK was confirmed (Figure 3A). However, the band in HE was much weaker compared with the HK (Figure 3A).

For the mRNA of G3PDH, the control, a DNA band corresponding to 432 bp was confirmed in cultured human corneal epithelial cells and keratocytes, as well as in cultured human skin fibroblast (Figure 3B).

Discussion

SP, one of the neurokinines, is a neuropeptide. It is thought to be a neurotransmitter of pain sense in the sensory nerve.[1] SP is also known to cause plasma effusion, vascular dilatation, and histamine release of mast cells in the peripheral tissue in the axon reflex.[17–22] In the domain of ophthalmology, it has been

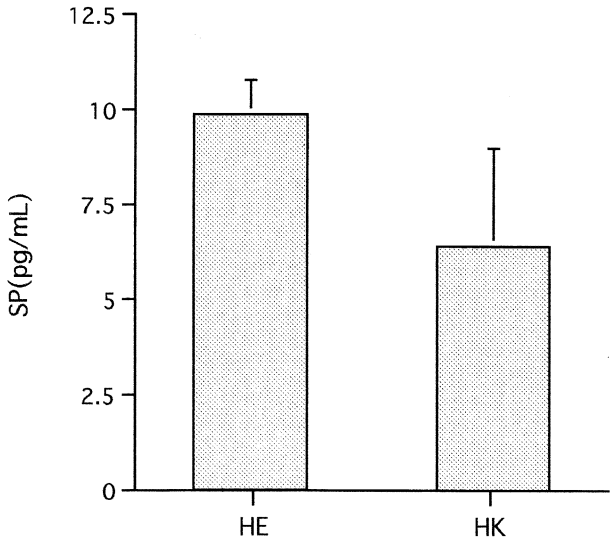

Figure 2. Assay of substance P (SP) synthesized by cultured human epithelial cells (HE) and cultured human keratocytes (HK). After having been cultivated for 2 days in serum-free Dulbecco's modified Eagle's medium, the supernatant in the culture dishes of HE and HK was collected. Enzyme-linked immunosorbent assay was performed on synthesized SP in the supernatant (n = 3).

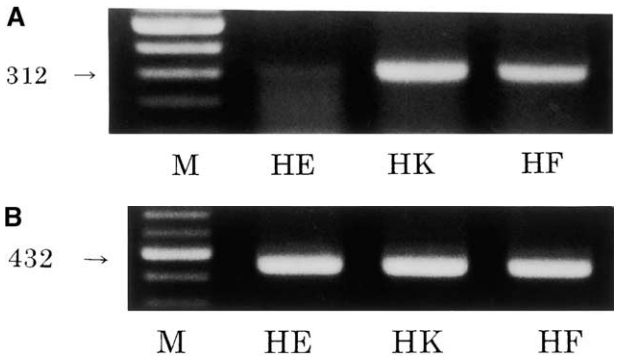

Figure 3. Messenger RNA (mRNA) expression of neurokinin 1 receptor (NK1R) and G3PDH by reverse transcription-polymerase chain reaction (RT-PCR) analysis. (**A**) The mRNA of NK1R was expressed in cultured human epithelial cells (HE) and cultured human keratocytes (HK) by RT-PCR analysis using specific primers. The size of the respective RT-PCR products amplified by using the primers was 312 bp. M: DNA marker. (**B**) The mRNA of G3PDH as the control was expressed in cultured HE cells and cultured HK by RT-PCR analysis using specific primers. The size of the respective RT-PCR products amplified by using the primers was 432 bp. M: DNA marker.

reported that SP is concerned with the immune function of the anterior chamber[23] in addition to its action to stimulate corneal epithelial cell proliferation.[2–5]

Three kinds of mRNA, α, β, and γ are synthesized as a result of splicing from preprotachykinin A gene, a precursor gene of SP.[24,25] Furthermore, it has been known that substance K (SK) is also produced from β and γ mRNA of these three kinds of mRNA.[24,25] As the primers for examining the mRNA of the SP used this time, we selected those capable of examining the expression of these three kinds of mRNA (α: 210, β: 264, and γ: 219 base pairs). As a result, γmRNA was confirmed in HE and β and γ mRNA in HK. This suggests that both HE and HK are likely to be producing SK in addition to SP. However, the results of our ELISA specifically measuring SP confirm that both HE and HK are producing only SP.

Regarding the characteristics of endogenous SP, it has been reported that the amount of SP produced by the human keratinocytes is increased by exogenous SP, and that the synthesis of SP is increased by the addition of adrenaline, acetylcholine, calcitonin gene-related peptide, and histamine.[9] Furthermore, endogenous SP is presumed to be associated with atopic dermatitis and acquired immune deficiency syndrome in human keratinocytes, lymphocytes, monocytes, and macrophages.[8–11] Our present experiment has confirmed that HE and HK cells produce SP. However, the samples used in this experiment are cultured cells, so further studies are needed on whether or not the corneal epithelial cells and keratocytes produce SP on their own in vivo, it is also desirable to perform studies on the physiological action of SP and its relation to corneal diseases.

In the present experiment, the mRNA of NK1R, an SP receptor, was detected in HE, suggesting that NK1R might be concerned with the effect of SP on the corneal epithelial cell. This may be taken as supporting the results of Nishida et al[2] and Araki-Sasaki et al.[6]

It is also possible that the SP produced by HE cells exerts effects in autocrine or paracrine fashion via NK1R to control the cellular function of the corneal epithelium.

On the other hand, to our knowledge, there has been no report that HK cells are influenced by SP. In our experiment, however, the expression of the mRNA of NK1R was confirmed in the HK, suggesting the influence of SP on HK.

In human skin fibroblast, SP's effect of stimulating proliferation via NK1R has already been reported in vitro.[15,26] Keratocytes in the healing process of corneal injury reportedly transform as do fibroblasts.[27] To our knowledge, there has been no report that human skin fibroblasts produce SP, but both types of cells are thought to have something in common. There appears to be much to investigate about the influence of SP on keratocytes in the healing process of corneal injury. Because the production of SP by HK was confirmed, it is possible that endogenous SP exerts effects in autocrine and paracrine fashion via NK1R to control cell function in human corneal epithelial cells.

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