Substance P and Its Metabolites in Normal Human Tears

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PURPOSE. To determine amounts and biochemical characteristics of substance P-like immunoreactivity (SPLI) in tears of normal human subjects.

METHODS. Forty-three healthy subjects (16 males and 27 females; age range, 17–80 years) participated. Ten microliters of unstimulated tears were collected with a micropipette from one eye of all subjects. Tear samples were partially purified by C-18 cartridges. SPLI concentrations in purified samples were measured by enzyme immunoassay (ELIA). For biochemical characterization of SPLI, tear extracts were fractionated by high-performance liquid chromatography (HPLC). Each fraction then was subjected to ELIA. To determine the metabolism of substance P in tears, synthetic substance P was incubated in medium containing pooled tears and then analyzed by HPLC with the detector set at a 210-nm wavelength.

RESULTS. The SPLI concentration in normal human tears was 306.0 ± 96.5 pg/mL (mean ± SD; range, 168–555 pg/mL). SPLI did not significantly vary by age or gender. HPLC analysis indicated that SPLI in tears consisted of five different substances and that substance P was converted to several fragments, including SP8-11, by enzymes present in tears.

CONCLUSIONS. Substance P, a normal component of human tears, presumably is released from the nerve endings in the ocular surface and converted to fragments by degradative enzymes in tears. (Invest Ophthalmol Vis Sci. 2002;43: 2622–2625)

The cornea is innervated by trigeminal sensory nerve fibers that contain several neuropeptides, including substance P and calcitonin gene-related peptide.1–5 When these neuropeptides are depleted with capsaicin, wound healing in the corneal epithelium is delayed.6 Substance P, both alone and in combination with other factors, such as insulin and insulin-like growth factor-1, promotes migration7,8 and proliferation9,10 of corneal epithelial cells. Recently, topically applied substance-P–derived peptide (FGLM peptide) combined with insulin-like growth factor-1,11 when applied to the eye, substance P produces miosis15,16 and may enhance intraocular inflammation and conjunctival hyperemia.17,18 Obviously, substance P present in tears of normal quiescent eyes does not provoke such a response. Degradative enzymes, such as enkephalinase, angiotensin-converting enzyme, and trypsin, are thought to cleave substance P released from nerve endings.19 Therefore, substance P sometimes is considered a precursor of a variety of closely related peptides exhibiting different actions. For example, SF1-11, which contains the N-terminal portion of substance P, is involved in the mechanism of neurogenic inflammation in allergic disorders,20 whereas C-terminal SF8-11 (FGLM peptide) influences the epithelial cell migration in the cornea.11 However, little is known about the metabolites of substance P in the cornea or in tears. This study was undertaken to determine concentrations and biochemical characteristics of substance P and its related peptides in tears of normal human subjects.

SUBJECTS AND METHODS

Subjects

Forty-three subjects (16 males and 27 females; age range, 17–80 years) participated in the study. Each participant underwent a thorough initial eye examination, including a slit lamp examination, Schirmer testing, and a cotton-thread test. All subjects had more than 10 mm of Schirmer strip wetting, and more than 15 mm of cotton-thread wetting. Normal corneal sensation (55 mm or greater) was confirmed by using a Cochet-Bonnet esthesiometer. Except for eight elderly subjects with age-related cataract, all subjects were free of any ocular disease. Subjects who wore contact lenses, had diabetes mellitus, or had a history of any ocular surgery were excluded from study. The principles of the World Medical Association’s Declaration of Helsinki were followed. Each subject received a full explanation of all procedures and provided informed consent for participation before the experiment. Approval for this investigation was granted by the Committee for the Protection of Human Subjects at Keio University School of Medicine.

Substance P–like Immunoreactivity Assay

Ten microliters of unstimulated tears were collected with a micropipette (Drummond Scientific, Broomall, PA) from one eye of all subjects. Tear samples were partially purified by C-18 cartridges (Sep-Pak; Waters, Milford, MA). Samples were diluted fivefold with 4% acetic acid and loaded onto C-18 cartridges (Sep-Pak; Waters, Milford, MA). After samples were washed with 3 mL of 4% acetic acid and loaded onto C-18 cartridges, SPLI was determined using the Substance P–like Immunoreactivity Assay.
TABLE 1. Substance P-like Immunoreactivity in Normal Human Tears

<table>
<thead>
<tr>
<th>Subjects</th>
<th>SPLI</th>
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<tbody>
<tr>
<td>Males ($n = 16$)</td>
<td>$275.5 \pm 76.7$ (148–417)</td>
</tr>
<tr>
<td>Females ($n = 27$)</td>
<td>$322.3 \pm 101.9$ (161–556)</td>
</tr>
<tr>
<td>Total ($n = 43$)</td>
<td>$306.0 \pm 96.5$ (148–556)</td>
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No significant difference was found between male and female subjects, although considerable variation was noted between individuals. Data are mean picograms per milliliters SPLI, with the range in parentheses.

tears was eluted with 3 mL ethanol-water-acetic acid (90:10:0.04, vol/vol/vol).20 A standard substance P solution (100 pg/mL; Sigma, St. Louis, MO) was used to estimate the extent of the recovery rate during this partial purification step.

The eluate was concentrated by evaporation and then reconstituted in 50 μL phosphate buffer. Immunoreactive substance P in purified samples was measured using an enzyme immunoassay (EIA) kit (Cayman Chemical, Ann Arbor, MI). The antiserum used in the assay, which was specific to the C-terminal portion of substance P, reacted with $SP_{2-11}, SP_{5-11}, and SP_{4-11}$, but not with $SP_{8-11}$ or $SP_{1-9}$. The final results are expressed as picograms per milliliter.

High Performance Liquid Chromatography
An HPLC system was used that consisted of a solvent delivery system (LC-10AD), a programmable detector (UV-VIS) set at 210 nm, a chromatography column (ODS-2; Whatman, Clifton, NJ) at a rate of 0.5 mL/min.21 Synthetic substance P, $SP_{2-11}, SP_{5-11},$ and $SP_{4-11}$, but not with $SP_{8-11}$ or $SP_{1-9}$. The eluate was concentrated by evaporation and then reconstituted in 50 μL HPLC-EIA buffer. These residues were subjected to identification of peaks.

Three 100-μL samples of pooled tears (each was collected from both eyes of five subjects) mixed with 400 μL aprotinin-EDTA were used for HPLC-EIA. The samples were purified by a C-18 cartridge, as just described. The purified samples, reconstituted in 10 μL HPLC solvent, were fractionated by HPLC (fraction size, 0.25 mL). Each fraction was concentrated by evaporation under nitrogen gas and then reconstituted in 50 μL EIA buffer. These residues were subjected to EIA.

To determine metabolism of substance P by degradative enzymes in human tears, three 50-μL samples of pooled tears (each was collected from one eye of five subjects) were used. Ten micrograms of synthetic substance P (Sigma) was incubated for 60 minutes at 37°C in 0.5 mL DMEM/Ham’s F-12 containing 50 μL pooled human tears. Substance P (10 μg) incubated in 0.5 mL DMEM/Ham’s F-12 alone served as the negative control. The fluids were purified by C-18 cartridge and then analyzed by HPLC with UV detection at 210 nm.

RESULTS
SPLI Concentration in Human Tears
The mean SPLI concentration in normal human tears was 306.0 ± 96.5 pg/mL (mean ± SD; range, 148–555 pg/mL; Table 1). SPLI concentrations in tears were not significantly associated with age or gender (Fig. 1).

To test reproducibility of the measurement, tear samples were collected twice on different days in 10 subjects (6 men and 4 women; age range, 24–27 years). The mean SPLI concentration in tears collected on the first day was 316.6 ± 99.3 pg/mL, whereas that on the second day was 299.5 ± 95.3 pg/mL, which was not significantly different.

Tear samples were collected from both eyes in 10 subjects (5 men and 5 women; age range, 24–27 years). Tears were collected from the right eye first and then from the left. The mean SPLI concentration in tears from right eyes was 267.1 ± 48.7 pg/mL, and in tears from left eyes was 272.7 ± 70.2 pg/mL, which was not significantly different.

HPLC-EIA of SPLI
HPLC-EIA indicated that SPLI in tears consisted of five different substances. A representative chromatogram is shown in Figure 2. The first peak, with a retention time shorter than that for $SP_{8-11}$, was assumed to be SP-sulfoxide.13,14 No peak for $SP_{8-11}$ was seen, possibly because the antibody used in the EIA system did not react with $SP_{8-11}$. The second to the fifth peaks of HPLC-EIA comigrated with authentic substance P, $SP_{2-11}, SP_{5-11},$ and $SP_{4-11}$, respectively. Substance P was the major SPLI in tears, although such fragments as $SP_{2-11}, SP_{5-11},$ and $SP_{4-11}$ were identified.

Metabolism of Substance P by Degradative Enzymes in Human Tears
HPLC analysis revealed that six peaks were present after substance P was incubated in a medium containing human tears. A
representative chromatogram is shown in Figure 3. The peaks were identified as SP sulfoxide, SP$_{8-11}$, substance P, SP$_{2-11}$, SP$_{5-11}$, and SP$_{4-11}$ (peaks 1–6, respectively). When substance P was incubated in medium alone, two small peaks (SP-sulfoxide and SP$_{8-11}$) were identified in addition to substance P (Fig. 4).

**DISCUSSION**

Our results showed substance P to be a component of tears obtained from normal human eyes. The mean SPLI concentration in normal human tears was 306.0 ± 96.5 pg/mL (range, 148–555). Although there was considerable variation between individuals, concentrations of SPLI in tears was not significantly associated with age or gender. Reproducibility of measurements in a subject for different days or eyes was reasonably good.

The concentration of SPLI in tears in the present study was higher than previously reported by Varnell et al.\textsuperscript{12} and Fujishima et al.\textsuperscript{13} Although all three studies used EIA to quantitate the concentration of SPLI, differences in the purification procedure and the antibody used in the assay may account for the discrepancy. Fehder et al.\textsuperscript{20} have reported that proteins in tears influence measurements of SPLI, and partial purification is therefore required to measure SPLI in samples containing differing amounts of various proteins.

HPLC-EIA analysis indicated that SPLI in tears consisted of at least five different substances. After SP-sulfoxide, a nonenzymatic oxidative product of substance P, and substance P itself, the third to the fifth peaks of HPLC-EIA comigrated with authentic SP$_{2-11}$, SP$_{5-11}$, and SP$_{4-11}$, respectively. As mentioned in the Methods section, the antiserum used in the assay was specific for the C-terminal portion of substance P. N-terminal fragments of substance P or C-terminal fragments smaller than SP$_{5-11}$ were not detected by the assay. Accordingly, synthetic substance P was incubated in a medium containing human tears, to determine metabolism of substance P by degradative enzymes in tears. HPLC detected the six peaks mentioned earlier. SP$_{8-11}$ was the major metabolite of substance P after incubation with human tears. These results suggest that SP$_{8-11}$ is present in normal tears. Our results also suggest that several enzymes in tears take part in degrading substance P, although nonenzymatic degradation and nonenzymatic oxidation occur to some extent. Candidate degradative enzymes include trypsin, dipeptidyl aminopeptidase IV, postproline-cleaving enzyme, and enkephalinase.\textsuperscript{16}

Enzymatic degradation of substance P released from nerve endings in tears appears to be biologically advantageous. The miotic effect of substance P would be avoided or attenuated by the degrading process.\textsuperscript{22}

N-terminal fragments of substance P including SP$_{5-9}$, which is involved in the mechanism of allergic inflammation,\textsuperscript{19} were not identified in normal human tears in the present study. It is of interest to know whether N-terminal fragments of substance P are present in tears of patients with severe allergic conjunctivitis, because elevated levels of substance P have been reported in tears of such patients.\textsuperscript{13} Nakamura et al.\textsuperscript{23} have shown that FGLM peptide, a sequence of four amino acids in the C-terminal of substance P, is the minimum sequence necessary to produce synergistic effects of substance P and insulin-like growth factor-1 on corneal epithelial wound healing. Notably, all metabolites of substance P detected in this study retained this minimum sequence. Therefore, degradation of substance P in tears may have a beneficial role, permitting maintenance of the ocular surface by exerting trophic effects of substance P while avoiding undesirable effects.

**References**


