Increased Levels of Diadenosine Polyphosphates in Dry Eye

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PURPOSE. To analyze the levels of the diadenosine polyphosphates Ap4A and Ap5A in tears, in a set of control subjects and in groups of symptomatic and nonsymptomatic persons with dry eye.

METHODS. Ninety-seven subjects participated in the study. The subjects were divided into five experimental groups: control subjects—symptomatic patients with normal tear secretion; symptomatic patients with low tear secretion; forced blink; and corneal mechanical stimulation provided by a gas esthesiometer. The Schirmer I test was used to measure and collect tear secretions from each subject. All samples were processed by high pressure liquid chromatography (HPLC) and their Ap4A and Ap5A levels determined.

RESULTS. The levels of Ap4A and Ap5A in tears were greater in all symptomatic patients than in control subjects, especially in symptomatic subjects with low tear secretion. Within the symptomatic subjects with normal tear secretion, significant differences in concentrations of Ap4A and Ap5A were found between men and women. In the forced blink experiments, concentrations of the Ap4A and Ap5A rose with increasing blink frequency. When the cornea was mechanically stimulated, the levels of Ap4A and Ap5A rose significantly during both moderate and high-flow rate tests.

CONCLUSIONS. The increased levels of Ap4A and Ap5A in tears of patients with dry eye allow these dinucleotides to be used as objective biomarkers in dry eye conditions. (Invest Ophthal-mol Vis Sci. 2006;47:4053–4058) DOI:10.1167/iovs.05-0980

The tear film constitutes a moist natural barrier that separates the eye from the external media. This consistent film is formed mainly by a triplet of aqueous, mucous, and lipid layers that provide the necessary equilibrium for maintaining the health of the ocular surface.1 The principal functions of this film are: (1) to keep the ocular surface wet and well lubricated; (2) to transfer the nutritional elements to the cornea, (3) to eliminate foreign matter and cellular debris generated on the ocular surface by the tear flow and the blink process; and (4) to act as the first line of defense against ocular surface infections.2

The balance between the composite layers of the tear film is critical, and when altered it can give rise to complications on the ocular surface. These complications are widely known as dry eye–related problems, the severity of which depends on the level of imbalance. Two causes of this disequilibrium are a deficiency in tear secretion caused by Sjögren’s syndrome, or tear film instability due to the wearing of contact lenses. Signs and symptoms of these complications can vary from patient to patient, often with little or no correlation between them. Patients may present with low tear secretion volume, ocular signs, and no ocular symptoms or with normal tear volume and ocular symptoms of dryness. This poor correlation is well known, having been frequently reported in the literature.3–5 This ambiguity in etiology and pathophysiology6 can make a precise diagnosis difficult.

Today, there are several questionnaires and tests designed to evaluate patients’ signs and symptoms. Among these tests, the National Eye Institute/Industry Dry Eye Workshop7 proposed the combined use of a validated questionnaire regarding symptoms, a test to evaluate ocular surface damage, measurement of tear instability, and the demonstration of tear hyperosmolarity.

More recently, the second National Eye Institute/Industry Workshop on Clinical Trials has given rise to new criteria for subjective and objective testing for dry eye, and several tests, both invasive and noninvasive, are now emerging.8 These tests are designed to identify and analyze biomarker profiles in tears to detect clearly whether an individual has dry eye. The identification of a stable molecule present in tears whose concentration changes with different dry eye conditions, would make diagnosis, follow-up, and the assessment of dry eye treatments both easier and more precise.

Pintor et al.9 have described the presence of a new family of compounds in the tear film, the diadenosine polyphosphates. These compounds, are naturally occurring dinucleotides, exhibiting both intracellular and extracellular physiological effects10,11. Formed by two adenosine molecules joined by a variable phosphate chain, they are abbreviated as ApnA (n = 2–7). Although the activity of these nucleotides in ocular tissues has not been fully investigated, it is known that they act through P2 receptors to modulate intraocular pressure in rabbits.12 Ap4A and UTP improve the rate of wound healing in the cornea of New Zealand White rabbits13. Ap4A, Ap5A, and Ap5P can stimulate tear secretion after single-dose topical application in rabbits.14

In this study, the levels of diadenosine polyphosphates in tear secretions were examined in a group of control subjects without any symptom of ocular dryness and with a normal volume of tear secretion, as well as two groups of dry eye symptomatic subjects, one with low tear secretion volume and another with normal tear volume. Secretion of the dinucleotides was also examined in forced blink experiments and after moderate and strong corneal mechanical stimulation using a gas esthesiometer. Analysis of the resultant data suggests that...
these substances, in particular Ap$_4$A, can act as a tear film molecular marker for dry eye states. Preliminary results have been reported in abstract form (Peral A, et al. IOVS 2003;44: ARVO E-Abstract 2494).

**METHODS**

**Subjects**

Ninety-seven subjects of both sexes (27 men and 70 women, of age ranging from 20 to 36 years with a mean age of 27 ± 1 years) participated voluntarily in the study. The research was in compliance with the tenets of the Declaration of Helsinki. The subjects signed an informed consent and were free to interrupt the session at any time. The McMonnies $^5$ test was performed to detect possible symptoms of ocular dryness.

As identified by the McMonnies test, all nonsymptomatic subjects ($n = 51$), had a Schirmer I test$^{19}$ result equal to or greater than 10 mm. This population was then divided into three groups: those who would participate in the forced-blink experiment ($n = 8$), those who would take part in the esthesiometer measurements ($n = 6$), and a control group ($n = 37$).

Symptomatic subjects ($n = 46$), again as identified by the McMonnies test, were divided into two groups: symptomatic subjects with normal tear secretion ($n = 34$), those who presented symptoms of ocular dryness and had a Schirmer I test result ≥10 mm; symptomatic subjects with low tear secretion ($n = 12$), those who exhibited symptoms but whose tear secretion measured with the Schirmer I test was ≤5 mm.

The distribution of subjects and McMonnies scores for the different groups are summarized in Table 1.

**Trials**

**Tear Collection.** Tear secretion was measured in all groups with the Schirmer I test. In control group and symptomatic subjects, the Schirmer strip was located on the temporal tarsal conjunctiva of the lower lid for 5 minutes with the eyes closed. Secretion was measured without any kind of stimulation.

**Forced-Blink Experiment.** Schirmer trips were located at the temporal tarsal conjunctiva of the lower lid of eight nonsymptomatic subjects with normal tear secretion. The patients were instructed to blink at 0, 12, 30, and 60 blinks per minute during 5 minutes.

**Esthesiometry Experiment.** Schirmer strips were located at the temporal tarsal conjunctiva of the lower lid of six nonsymptomatic subjects with normal tear secretion for 5 minutes after the corneal stimulation. The cornea was mechanically stimulated with a gas esthesiometer.$^{17}$ Three gas pulses, each lasting 3 seconds, were applied sequentially (interval, <0.5 seconds) to the center of the cornea. Two flow rates of mechanical stimulation were applied: moderate (170 mL/min) and high (260 mL/min) flow.$^{18}$ Subjects were asked to avoid blinking during the application of the three gas pulses.

In all groups, the volume of tears, measured as millimeters of moistened Schirmer strip, was recorded and the strips placed in tubes (Eppendorf, Fremont, CA) containing 500 μL of ultrapure water. The tubes were then frozen until HPLC analysis was performed.

**Sample Processing and HPLC Analysis**

Schirmer strips were collected, placed in Eppendorf tubes containing 500 μL of ultrapure water, and strongly vortexed for 5 minutes. The strips were carefully rinsed and the liquid in the tube was heated in a 100°C bath for 20 minutes to precipitate proteins. The tubes were centrifuged at 4000 rpm for 50 minutes to form a pellet of the proteins. Diadenosine polyphosphates are resistant to this treatment, as demonstrated by Pintor et al.$^{19}$ The supernatants were chromatographed (SEP-PAK Accell QMA cartridges; Waters, Milford, MA). Briefly, 250 μL of the supernatant was passed through the cartridges which had been equilibrated with 3 mL of ultrapure water. The elution of the nucleotides and dinucleotides was performed by applying 1 mL of a solution containing 0.2 M KCl and 0.1 M HCl, and the samples were neutralized with KOH. The eluents were then injected at a volume of 10 to 100 μL into the HPLC for analysis.

Determination and quantification of diadenosine polyphosphates were performed by HPLC. The chromatographic system consisted of a isocratic HPLC pump (model 1515; Waters), a dual absorbance detector (2487; Waters), and an injector (Reodyne, Rhonert Park, CA), all managed by the software (Breeze) and a column from Waters (15 cm length, 0.4 cm diameter; Novapack C18).

The system was equilibrated overnight with the following mobile phase: 0.1 M KH$_2$PO$_4$, 2 mM tetrabutyl ammonium, and 17% acetonitrile (pH 7.5).$^{19}$ Detection was monitored at a 260-nm wavelength, and all the peaks identified as putative dinucleotides were taken for phosphodiesterase treatment. Phosphodiesterase from Crotalus durissus, from Sigma-Aldrich (St. Louis, MO; EC 3.1.15.1) at a concentration of 0.3 U/mL was incubated for 10 minutes with the corresponding putative dinucleotide. The digestion products were analyzed by HPLC. Peaks were transformed into concentrations by means of external commercial diadenosine polyphosphates standards of known concentrations.

**Statistical Analysis**

The data are presented as the mean ± SD of results of the experiments. Data were analyzed with Student’s $t$-test, and significance was set at $P < 0.05$.

**RESULTS**

**Ap$_4$A and Ap$_5$A Levels for the Control Group and Symptomatic Subjects**

Pintor et al.$^9$ identified the presence of diadenosine polyphosphates in human tears; however, little is known about the levels of concentration in the tears of individuals presenting with ocular dryness. The levels of these substances in both normal and pathologic eyes were therefore investigated in the present study.

Two peaks were tentatively identified as Ap$_4$A and Ap$_5$A, according to the HPLC retention times (Fig. 1A). To confirm that these peaks corresponded to Ap$_4$A and Ap$_5$A, they were individually collected and treated with phosphodiesterase from Crotalus durissus (Figs. 1B, 1C). The treatment of the putative Ap$_4$A produced two peaks after the enzyme action, identified

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<th>Table 1. Distribution of McMonnies Scores in the Different Groups</th>
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The concentrations of these dinucleotides in the symptomatic group were female. The potential divergence gives rise to the possibility that there may be a hormonal factor involved, which may bear further investigation. These mentioned divergences may permit quantification of the concentrations of the two dinucleotides in the samples (Fig. 1D). In the control group, the appearance of the hydrolytic products AMP and ATP, whereas the putative Ap5A yielded AMP and Ap4. (C) Rechromatography of the Ap5A peak shown in (A) after treatment with phosphodiesterase. The reduction in the dinucleotide peak is concomitant with the increase in the blink frequency. The augmented concentrations of Ap5A and Ap4 for the different spontaneous blink frequencies paralleled an increase in tear secretion. These results are shown in Figure 4 in which an increase in the Schirmer scores occurred with the increase in frequency of the forced blink.


The forced blink experiments demonstrated a clear increase in the tear concentrations of Ap5A and Ap4 with decreasing blink frequency. This increase could be due to the shear stress produced by the eyelids on the corneal surface. Srinivas et al. described how the mechanical action of the eyelids on the ocular surface can provoke a shear stress at the corneal epithelial cells and subsequently a release of intracellular substances to the tear film, including nucleotides and dinucleotides.

One question that arises is whether the release of nucleotides open the possibility of performing future studies to see whether hormonal factors could be responsible for such variability.


Tsubota et al. reported that patients with dry eye can increase their blink rate to compensate for tear instability. Accordingly, in this study, the effect of forced blinking on levels of diadenosine polyphosphates in the tear film was examined. Eight subjects with no symptoms of ocular dryness and a normal volume of tear secretion participated in the experiment. Variations in Ap5A and Ap4 concentrations in their tears were investigated for four different blink frequencies: 0, 12, 30, and 60 blinks per minute, with 0 blinks per minute (eyelids closed) being considered the control for this experiment.

The chromatographic analysis revealed substantial changes in the concentrations of Ap5A and Ap4A with differing blink frequency (Fig. 3A). When these peaks were transformed into concentrations, the levels of Ap4A rose from 0.109 ± 0.081 μM for 0 blinks/min to 2.853 ± 0.394 μM at 60 blinks/min (Fig. 3B). An almost identical trend was observed for Ap5A, with concentrations rising from 0.041 ± 0.050 μM (0 blinks/min) to 2.091 ± 0.065 μM at 60 blinks/min (Fig. 3B). It was possible to observe a gradual increase in the dinucleotide concentration concomitant with the increase in the blink frequency.

The augmented concentrations of Ap5A and Ap4 for the different blinking frequencies paralleled an increase in tear secretion. These results are shown in Figure 4 in which an increase in the Schirmer scores occurred with the increase in frequency of the forced blink.
is simply a product of this shear stress, or whether it is trig-
ergered by mechanical stimulation of corneal nerve terminals. To
investigate this, a gas esthesiometer was used to reproduce the
mechanical stimulation.

Six subjects participated in this part of the study, all show-
ing a normal volume of tear secretion and none presented
symptoms of ocular dryness according to the McMonnies test.

Tears were collected after application of the mechanical stim-
uli, and the diadenosine polyphosphates were isolated and
quantified by HPLC.

The concentrations of diadenosine polyphosphates were
found to be statistically independent (95% confidence) of the
intensity of the mechanical stimulus applied (Fig. 5). Both
moderate (170 mL/min) and high (260 mL/min) mechanical
stimulation of the corneal nerves, resulted in a significantly
greater amount of dinucleotides than that in the control sub-
jects (n = 6). The moderate mechanical stimulus produced
tear concentrations of 0.170 ± 0.012 and 0.058 ± 0.073 μM
tration for the high mechanical stimulus were 0.163 ± 0.048

and 0.065 ± 0.007 μM, respectively (Fig. 5). Even the moder-
ate mechanical stimulus, which may be of the same intensity as
provoked by the eyelids during the blinking process and not
strong enough to stimulate the majority of corneal polymodal
and mechanonociceptors, resulted in increased concentrations
of diadenosine polyphosphates in tears.

**DISCUSSION**

The results show that patients with symptomatic dry eye with
a reduced tear secretion have greatly elevated levels of the
adenosine dinucleotides Ap4A and Ap5A in their tears, being
increased 100- and 345-fold for Ap4A and Ap5A, respectively.
Symptomatic patients with normal tear secretion also demon-
strated an increase in diadenosine polyphosphate concentra-
tions, although not as marked as those with low tear produc-
tion. These concentrations of Ap4A and Ap5A were increased 5-
and 1.5-fold, respectively.

Furthermore, a difference between the sexes was evident.
Symptomatic women with normal tear secretion presented
higher values of diadenosine polyphosphates than did similar
men. However, there was no significant difference between
the sexes in the control group. This result suggests that the sex
of a patient should be taken into account as well as the
measure of diadenosine levels in tears, in a specific test to
determine borderline dry eye disease.

**FIGURE 4.** Volume of tear secretion measured by Schirmer I test for
the different frequencies (bpm) of blinking. The Schirmer test results
increased with the increase in the blink rate. Data from both eyes were
pooled and are expressed as the mean ± SD. **P < 0.0005 for 30 bpm
and 60 bpm, paired t-test.

**FIGURE 5.** Effect of mechanical stimulation of the cornea with a gas
esthesiometer on diadenosine polyphosphate levels in tears. Didade-

osine polyphosphates increased in concentration in tears after both
moderate and high corneal mechanical stimulation when compared
with the control. Data from both eyes were pooled and are expressed
as the mean ± SD. *P < 0.05, paired t-test.
Because it has been shown that Ap4A and Ap5A stimulate tear secretion,\(^1\)\(^4\) it was unexpected to see patients with low tear secretion having increased concentrations of these dinucleotides. It appears that although Ap4A and Ap5A are plentiful, they are not able to stimulate lacrimation, and it could be that in those patients, dry eye is a consequence of a lacrimal gland malfunction. The mechanism of action of the dinucleotides is not fully understood. Possibly, they increase tear production by stimulating corneal and conjunctival water and electrolyte production, by causing vasodilatation of conjunctival blood vessels, or by inducing conjunctival goblet cell secretion or increasing meibomian gland secretion. In aqueous-deficiency dry eye, one or more of these processes could be defective, and thus tear volume could be decreased even in the presence of increased levels of Ap4A and Ap5A.

In the symptomatic normal tear secretion dry eye group, tear composition, rather than volume, could be altered causing the symptoms. Dinucleotides present in tears could be stimulating tear secretion, and although the tears are defective, they are produced in normal amounts because of the increased levels of Ap4A and Ap5A. More work is needed to clarify this point.

The mechanism by which diadenosine polyphosphates and other nucleotides such as ATP enter the extracellular fluid has not yet been identified. It is possible, as occurs in the central nervous system, that nucleotides are liberated from nerve terminals, but there is evidence that nucleotides can be transported out of cells. Epithelial cells,\(^2\)\(^5\)\(^-\)\(^2\(^4\)\) and in particular ocular epithelial cells,\(^2\(^5\)\) use different transport mechanisms as a regulated procedure for nucleotide release. The ATP binding cassette (ABC) transporter, the cystic fibrosis transmembrane conductance regulator (CFTR), or glycoprotein P have been proposed as elements involved in the release of nucleotides.\(^2\(^6\)\)\(^-\)\(^2\(^8\)\) Gomes et al.\(^2\(^9\)\) have described that ATP leaves corneal endothelial cells by means of connexin hemichannels when these cells are stimulated mechanically. It would therefore not be surprising that a mechanism of release takes place, although experiments to test this are outside the scope of the present study.

The forced blinking experiments replicate a natural shear stress, and the elevated levels of dinucleotides as a result of increased frequency of blinking are indicative of mechanically stimulated release. It is known that ocular surface conditions can affect the pattern of blinking and that patients with dry eye–related problems can increase their blink rate to compensate for tear instability or deficiency. Tsubota et al.\(^2\(^1\)\) described a blink rate of \(\frac{3}{4}\) blinks per minute in subjects with dry eye, compared with a normal blinking rate of 10 to 15 blinks per minute, which has been described as an essential, involuntary action for the protection of the ocular surface.\(^3\(^0\)\)\(^-\)\(^3\(^1\)\)

The elevated levels of dinucleotides in patients with dry eye is compatible with an increased rate of blinking. Patients with dry eye and low tear secretion had values of Ap4A and Ap5A of 1.14 and 0.91 \(\mu\)M respectively, which were comparable to levels recorded in normal subjects forced to blink at 30 per minute. This suggests that an increase in dry eye symptoms followed by an increase of the blinking rate could also produce an enhancement of the Ap4A and Ap5A tear concentrations, and indicate that the appearance of these dinucleotides depends only on the rate of mechanical stimulation, and seems to be independent of tear secretion.

The relationship between the amounts of diadenosine polyphosphates and the blink rate, probably due to the friction of the eyelids on the ocular surface, seems to be clear. The gas esthesiometer\(^3\(^2\)\) permitted us to apply mechanical stimuli with air at different intensities, close or over the stimulation threshold of corneal nerves. This reveals whether these compounds are released as a consequence of mechanical corneal nerve stimulation.

Acosta et al.\(^1\(^8\)\) described that only strong stimulation (high mechanical intensity, chemicals, or severe cold) of corneal nerves increases tear secretion, which explains that chiefly stimulation of polymodal corneal sensory nerves evokes reflex tear secretion. Although both moderate and high mechanical stimuli stimulate corneal nerves, high-intensity stimuli recruits a larger population of nerves.\(^5\(^2\)\) The esthesiometer experiments performed showed a small but significant increase in diadenosine polyphosphate levels after both moderate and strong mechanical stimuli. The lack of difference between the two mechanical stimuli indicates that at least a part of the dinucleotide release is independent of neural stimulation and should respond to the effect of the mechanical force applied to the corneal epithelium. Acosta et al.\(^1\(^8\)\) reported that a strong stimulus increases tear secretion and also evokes a sensation of irritation that may induce a reflex tear secretion and blinking. This sensation appears more frequently in patients with dry eye and therefore the frequency of blinking is increased.\(^2\(^1\)\) It is apparent that the blinking process provides ocular protection and contributes to spreading the tear film over the ocular surface. If there is a deficient or unstable tear film, the evaporation rate as well as the necessity of blinking increase. The increase in blinking can induce a shear stress on the ocular surface and, moreover, this fact can lead to raised levels of nucleotides in tears. It has been suggested that these substances, Ap4A and Ap5A, have a role in the stimulation of tear secretion,\(^1\(^4\)\) and it appears that they are naturally released to try to increase the tear volume/quality stimulating P2Y2 receptors present in meibomian and accessory glands.\(^3\(^5\)\) Moreover, because these substances are in any case increased in dry eye, we would like to suggest that these dinucleotides may be used as markers for dry eye conditions.

In summary, the levels of diadenosine polyphosphates in tears were analyzed in different groups of patients with dry eye. The group of symptomatic subjects with low tear secretion as well as the symptomatic subjects with normal tear secretion presented higher levels of diadenosine polyphosphates in tears than did the control subjects. In addition, significant differences were found between men and women and in this symptomatic group, the levels being higher in women. Besides symptomatology, other possible aspects related to dry eye, such as blinking frequency and corneal sensitivity, have been taken into account. While the dinucleotide levels were significantly increased concomitantly with the increase in the blink rate, the contribution from the corneal nerve stimulation was not so important.

These stable molecules could be an objective parameter for scoring the severity of dry eye, the follow-up of the disease, and to determine the efficacy of treatment.

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References


