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Intra- and inter-day variation of cytokines and chemokines in tears of healthy subjects

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ABSTRACT

Tear levels of certain cytokines/chemokines can potentially serve as biomarkers for dry eye and other ocular surface diseases if they remain stable from day-to-day in healthy eyes. The aim of this study was to determine the normal intra- and inter-day variation of selected tear cytokines/chemokines. Tear samples from 24 young, healthy adults were collected 11:00 AM–1:00 PM (mid-day) and 5:00–7:00 PM (evening) on three non-consecutive days. Concentrations of 18 cytokines/chemokines (EGF, eotaxin, CX3CL1/fractalkine, GM-CSF, IFN-γ, IL-10, IL-1β, IL-13, IL-17A, IL-1RA, IL-5, IL-6, CXCL8/IL-8, IL-9, CXCL10/IP-10, CCL5/RANTES, TNF-α, and VEGF) were measured by multiplex bead analysis. Ocular surface disease was ruled out by clinical tests. A random-effects ANOVA model was used to evaluate intra- and inter-day effects on cytokine/chemokine levels. Repeatability of intra-subject inter-day measurements was assayed by coefficient of variation. Ten out of the 18 molecules had detectable tear levels in >50% of the subjects. Of those, only IL-10 and IL-1β levels had significant inter-day variations. EGF, CX3CL1/fractalkine, CXCL10/IP-10, and VEGF were consistently higher in the evening compared to the mid-day measurements. EGF, CXCL10/IP-10, VEGF and CXCL8/IL-8had good intra-subject reproducibility. In conclusion, tear cytokines/chemokines can be measured reproducibly over time, with most not having significant inter-day variability. Some varied significantly depending upon the time of tear collection, and these variations should be taken into account when comparisons are made. The good intra-subject reproducibility for EGF, CXCL10/IP-10, CXCL8/IL-8, and VEGF indicates that these molecules could potentially serve as biomarkers of ocular surface disease.

1. Introduction

Cytokines and chemokines are pleiotropic molecules secreted by inflammatory cells, epithelial and endothelial cells, fibroblasts, and others. These endogenous chemicals affect and regulate a large number of biological phenomena, such as haematopoiesis, angiogenesis, degranulation, phagocytosis, growth and development, and immune cell trafficking (D'Ambrosio et al., 2003; Dinarello, 2007). Their presence in normal tears has been described in several studies (Carreño et al., 2010; Nakamura et al., 1998; Sack et al., 2007; Sonoda et al., 2006), and they are critical to numerous functions of the ocular surface, such as antimicrobial defense, wound healing and inflammatory responses (Cook, 2004; Leonardi et al., 2006; Nakamura et al., 1998). Also, numerous studies have characterized the role of different cytokines and chemokines in various ocular surface pathologies (Barton et al., 1997; Cook et al., 2001; Enriquez-de-Salamanca et al., 2010; Leonardi et al., 2006). These include the maintenance, coordination, and persistence of inflammatory processes, especially in dry eye disease (DED) and ocular allergies. Cytokines and chemokines such as interleukin (IL)-1β, IL-6, CXCL8/IL-8, and tumour necrosis factor (TNF)-α are increased in tears of patients with inflammatory ocular surface diseases (Balasubramanian et al., 2012; Enriquez-de-Salamanca et al., 2010; Fukuda et al., 1997; Jones et al., 1994; Lam et al., 2009; Massingale et al., 2009; Pflugfelder et al., 1999; Shoji et al., 2006; Solomon et al., 2001; Tishler et al., 1998; Yoon et al., 2007). Several studies have likewise shown that the levels of some of these molecules were correlated with various clinical parameters (Enriquez-de-Salamanca et al., 2010; Jones et al., 1994;

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Lam et al., 2009; Pflugfelder et al., 1999; Solomon et al., 2001; Tishler et al., 1998; Yoon et al., 2007). The specific role for each of these molecules in the different ocular surface inflammatory diseases is out of the scope of this study and is reviewed elsewhere.

Thus it has been proposed that the levels of cytokines and chemokines in tears can be used as potential biomarkers for ocular surface diseases. A premise of this proposal is that tear cytokine/chemokine levels must not vary significantly during the day or from day-to-day in healthy eyes. A study by Huang et al. of healthy subjects and dry eye patients showed good inter-visit repeatability for 23 of 43 tear film proteins, including some cytokines and chemokines (Huang et al., 2012). However, there are several studies that indicate differences in the concentration of cytokines and chemokines in salivary fluid and blood depending on the time of day (Rudney, 1995; Thomas et al., 2009). Also, some studies have shown differences associated with circadian rhythms in the secretion of these molecules by some cell types (Holzheimer et al., 2002; Keller et al., 2009; Pan et al., 2002; Petrovsky and Harrison, 1998; Vgontzas et al., 2003). Recently, Markoulli et al. (2012) reported intra-day variations of metalloproteinase (MMP)-9, tissue inhibitor of metalloproteinase (TIMP)-1, neutrophil gelatinase-associated lipocalin (NGAL), and total protein content (TCP) tear levels. However, to date there is little data about the natural intra-day variation that exists in the concentration of cytokines and chemokines in tears. To our knowledge, only Uchino et al. (2006) have addressed this issue. They found that IL-1β, IL-6, IL-10, IL-12p70, and TNF-α varied through the day, with concentrations slightly increased in the morning (about 12:00 AM (i.e noon)) and late in the day (9:00 PM–12:00 PM (mid-night)).

The aim of this study was to determine if tear levels of a wide panel of cytokines and chemokines in samples obtained from healthy subjects varied depending on the time of the day and/or on different days. Adequate knowledge of the physiological variations, such as intra-day changes and inter-day variations is necessary for valid comparison of tear cytokine levels between healthy and diseased eyes and in diseased eyes at before and after therapy.

2. Materials and methods

2.1. Subjects

This study was approved by the Institutional Review Board of IOBA and the Ethics Committee of the University of Valladolid and followed the tenets of the Declaration of Helsinki. Written consent was obtained from all subjects after explanation of the protocol. The criteria used for selecting subjects were age between 18 and 30 years old, under no systemic or ocular medication, with no previous history of systemic or ophthalmic disease known to affect tear production, no ophthalmic surgeries within the last three months, and no contact lens use. The subjects to be included in the study were required to have no ocular symptoms (Ocular Surface Disease Index – OSDI – score $\leq$12 points) (Schiffman et al., 2000) and results within normal limits in at least two of the following DED tests: tear film break-up time (TBUT) $\geq$7 s, negative fluorescein corneal staining, phenol red thread test $\geq$20 mm test, and Schirmer's test without topical anaesthesia $\geq$5 mm in 5 min.

2.2. Ophthalmic evaluation

To rule out ocular surface disease in the subjects the following ocular surface evaluations were performed on the inclusion visit: (1) The OSDI questionnaire was used to assess the presence or absence of symptoms of dry eye. This test was scored on a scale of 0–100. (2) Tear stability was evaluated by measuring TBUT. Fluorescein strips (Fluorets, Chauvin, Aubenas, France) previously wetted with 0.9% sodium chloride were gently applied to the inferior fornix. After three blinks, the time lapse between instillation of fluorescein and the appearance of the first randomly distributed dry spot was measured, and the mean of three measurements was recorded (Lemp, 1995). (3) Corneal integrity was evaluated by assessing fluorescein staining applied with strips. Corneal staining was scored following the Oxford Scheme (score 0–5) (Bron et al., 2003). (4) Tear production was measured by the Schirmer's test without topical anaesthesia. One sterile strip (Schirmer Tear Test Strips, 5 × 35 mm; Alcon Laboratories, Inc., Fort Worth, TX, USA) was placed in the lateral canthus of the inferior lid margin of both eyes (Halberg and Berens, 1961), and the subjects were asked to maintain closed eyes during the test. After 5 min the length of wetting of the strip was measured in millimetres. (5) Tear production was also measured by the phenol red thread test (Zone Quick Test; Menicon Ca, Ltd., Nagoya, Japan). The thread was placed over the lateral canthus of the eye and read after 15 s.

2.3. Tear sample collection

Subjects were evaluated twice per day between 11:00 AM and 1:00 PM (mid-day) and between 5:00 PM and 7:00 PM (evening), over three non-consecutive days with 2–5 day intervals, (i.e. a total of six visits). During the visits, the average relative humidity (±standard error of the mean) in the examination room was 31.8 ± 1.4% (range 23.0%–38.0%), and temperature was 19.3 ± 0.3 °C (range 18.0 °C–21.0 °C). These measurements corresponded to the average humidity in Valladolid (Cascos Maraña, 1982) and to a comfortable indoor temperature (Anonymous, 1997).

In each of the six visits, four microlitres of unstimulated tears were collected non-traumatically with a capillary tube (Drummond, Broomal, PA, USA) from one eye (randomly selected) as previously described (Enriquez-de-Salamanca et al., 2010). Tear sample volume (4 μl) collected was chosen on the basis that total basal tear volume in normal healthy subjects is 7.0–9.2 μl (Mishima et al., 1966; Kuppens et al., 1992) and therefore, these 4 μl would be already available without having to induce reflex production. Samples were obtained from the lateral canthus with extreme care not to touch the conjunctiva in order to avoid tear reflex. Subjects were allowed to blink normally during the sample obtaining. If any significant reflex tearing during the collection was observed, samples were discarded, and tear collection was resumed after a break of 5 min. Tear samples were placed into collection tubes containing 16 μl of Beadlyte Cytokine Assay buffer (Upstate-Millipore, Watford, UK) in a final volume of 20 μl (1/5 tear sample dilution). The collected tears were frozen at −80 °C and stored until measurement.

2.4. Analysis of tear cytokine/chemokine levels

Cytokine and chemokine levels in tear samples were determined by a multiplex bead analysis (Milliplex 18x-MPXHCYTO-60 Human Cytokine/chemokine panel, Millipore, Watford, UK). Levels of the following 18 molecules were measured: epidermal growth factor (EGF), eotaxin, CX3CL1/fractalkine, granulocyte-monocyte colony stimulating factor (GM-CSF), interferon (IFN)-γ, interleukin (IL)-1β, IL-5, IL-6, CXCL8/IL-8, IL-9, IL-10, IL-13, IL-17A, IL-1 receptor antagonist (IL-1RA), interferon inducible protein CXCL10/(IP)-10, CCL5/RANTES, TNF-α, and vascular endothelial growth factor (VEGF). Each diluted tear sample (10 μl) was incubated with antibody-coated capture beads overnight under agitation at 4 °C. After washing, the beads were further incubated with biotin-labelled anti-human cytokine and chemokine antibodies for 45 min, followed by streptavidin phycoerythrin incubation for 30 min. Finally, the beads were washed and analysed in a Luminex