Analytical Biochemistry 496 (2016) 30–34

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Absolute quantification of human tear lactoferrin using multiple reaction monitoring technique with stable-isotopic labeling

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ARTICLE INFO

Article history:
Received 8 October 2015
Accepted 10 December 2015
Available online 21 December 2015

Keywords:
Multiple reaction monitoring
Tears
Lactoferrin
Prostate cancer

ABSTRACT

The mass spectrometry technique of multiple reaction monitoring (MRM) was used to quantify and compare the expression level of lactoferrin in tear films among control, prostate cancer (CaP), and benign prostate hyperplasia (BPH) groups. Tear samples from 14 men with CaP, 15 men with BPH, and 14 controls were analyzed in the study. Collected tears (2 μl) of each sample were digested with trypsin overnight at 37 °C without any pretreatment, and tear lactoferrin was quantified using a lactoferrin-specific peptide, VPSHAVVAR, both using natural/light and isotopic-labeled/heavy peptides with MRM. The average tear lactoferrin concentration was 1.01 ± 0.07 μg/μl in control samples, 0.96 ± 0.07 μg/μl in the BPH group, and 0.98 ± 0.07 μg/μl in the CaP group. Our study is the first to quantify tear proteins using a total of 43 individual (non-pooled) tear samples and showed that direct digestion of tear samples is suitable for MRM studies. The calculated average lactoferrin concentration in the control group matched that in the published range of human tear lactoferrin concentration measured by enzyme-linked immunosorbent assay (ELISA). Moreover, the lactoferrin was stably expressed across all of the samples, with no significant differences being observed among the control, BPH, and CaP groups.

The tear film (TF) is the outermost thin liquid layer covering the ocular surface. It is a unique body fluid that is predominantly composed of mucins, proteins, lipids, and salts, and it serves multiple functions in order to maintain the health of the eye. The production of tears and their constituents is highly regulated, with both neuronal and hormonal influences [1–3]. The protein components of tears have been mined for their potential use as biomarkers of disease. With the advance of proteomic techniques, potential tear biomarkers have been identified for various ocular conditions, including diseases such as allergic conjunctivitis [1], allergy with corneal lesions [2], blepharitis [3], conjunctivochalasis [4], keratoconus [5], mycotic keratitis [6], pterygium [7], thyroid-associated orbitopathy (TAO) [8], and dry eye syndrome, and for systemic diseases such as clinically isolated syndrome (CIS) (a first neurological episode caused by inflammation/demyelination in one or more central nervous system sites) [9], cancers [10], and diabetes [11].

Abbreviations used: TF, tear film; ELISA, enzyme-linked immunosorbent assay; MRM, multiple reaction monitoring; CaP, prostate cancer; BPH, benign prostate hyperplasia; FA, formic acid; ACN, acetonitrile; TFA, trifluoroacetic acid; CV, coefficient of variation; TAO, thyroid-associated orbitopathy; CIS, clinically isolated syndrome; mRNA, messenger RNA.

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http://dx.doi.org/10.1016/j.ab.2015.12.006
0003-2697/

Although discovery of tear biomarkers has generated significant findings, tear protein quantification is still a challenge due to the limited basal tear volume that can be collected (usually < 10 μl). The enzyme-linked immunosorbent assay (ELISA) is a common technique used for protein quantitation. However, it has two main disadvantages, particularly for tear protein quantification. At this time, most commercial ELISA kits are developed to target serum or plasma proteins; therefore, tear research groups likely need to develop their homemade ELISA kits using available antibodies, which is time-consuming. In addition, each ELISA test will target only one specific protein, leading to the requirement for a large sample volume to validate and quantify the vast field of newly discovered potential tear biomarkers. This is a major hindrance for tear biomarker research because only small tear volumes can be collected from each individual. As a result, an alternative protein quantitation method is needed for tear protein quantitation.

Multiple reaction monitoring (MRM) using isotopically labeled peptides has been proposed as a good alternative [12]. In this study, a series of isotopically labeled heavy and natural/light synthetic peptide standards was used to generate a calibration curve. Based on the calibration curve, the concentration of target peptides in a wide dynamic range of 10^3–10^4 can be detected [13,14]. Compared with ELISA, the MRM technique has two advantages: it does not require the use of antibody, thereby eliminating the time required for antibody development, and it is capable of simultaneously quantifying up to 40 peptides. In ocular pharmacokinetic studies, MRM has been successfully used to absolutely quantify amphotericin B [15] and natamycin [16] treatments for fungal keratitis in rabbit tears as well as besifloxacin (an antibacterial agent for ophthalmic infections) in human tears [17]. This technique has also successfully quantified five tear proteins (lactoferrin, lysozyme, prolactin-induced protein, lipocalin 1, and proline-rich protein 4) in denatured pooled tears collected from contact lens wearers ($n = 3$) and non-contact lens wearers ($n = 4$) [18].

In terms of prostate cancer (CaP) biomarker research, the tear film is of particular interest because both the prostate gland and the major tear-producing gland, the lacrimal gland, are androgen regulated. In addition, significant low levels of lactoferrin protein expression were observed in tumor tissues and in sera from CaP patients [19]. As a result, in this study we used MRM to quantify and compare tear lactoferrin in individual (not pooled) tears collected from 14 men with CaP, 15 men with benign prostate hyperplasia (BPH), and 14 controls, which to our knowledge is by far the largest tear sample size ($n = 43$) tested using this technique.

Materials and methods

Participants

A total of 43 male participants (48–93 years of age) were recruited for this study; of this sample, 14 men were control subjects (no prostatic conditions), 15 were BPH patients (determined by biopsy examination), and 14 were CaP patients undergoing radical prostatectomy. Ethics approval for tear sample collection was obtained from the South Eastern Sydney Area Health Service ethics committee, and informed consent was obtained from all participants prior to sample collection.

Tear collection

All tear samples were collected in the morning. Tear samples from subjects with BPH and CaP were collected just prior to surgery. The tear sample collection followed the method previously published by our group [20]. Briefly, 5–10 μl of basal tears was collected by placing a BLAUBRAND intraMark micropipette (Brand, Germany) gently onto the corner of the interior lower lid margin of the tear meniscus for 5–10 min. The collected tears were directly expelled into Eppendorf tubes and then frozen at −80 °C until used.

Preparation of tears for MRM analysis

We simplified the method used by Masoudi and coworkers [18]. Here, 2 μl of individual tears was first diluted with MilliQ water to 20 μl and digested with 1 μl of sequencing-grade modified trypsin (Promega, Madison, WI, USA) overnight at 37 °C. The reaction was stopped by adding 5 μl of 5% formic acid (FA) (final ~ pH 3.0), and the digested samples were purified using C18 (200 μl) Stage Tips (Proxeon, Odense, Denmark) as per the manufacturer's instructions. Briefly, each Stage Tip was initialized with 20 μl of 50% (v/v) acetonitrile (ACN) and 5% (v/v) FA and then reequilibrated with 20 μl of 5% FA. The sample was loaded into the Stage Tip and slowly passed through the tip to allow peptide binding to the C18 column. The tip was washed with 20 μl of 5% (v/v) FA twice, and then the sample was eluted into a clean Eppendorf tube with 20 μl of 80% (v/v) ACN and 5% (v/v) FA. The eluate was then dried completely in a CentriVap Centrifugal Vacuum Concentrator (Labconco, USA) and frozen at −80 °C until used.

Preparation of peptide standards

Both lyophilized synthetic isotopically labeled peptide VPSHAVVA[R] containing [^{13}C,^{15}N]arginine (heavy peptide, molecular weight of 945.2) and the natural peptide VPSHAVVAR (light peptide, molecular weight of 935.2) were purchased and diluted in 5% (v/v) trifluoroacetic acid (TFA, Sigma). VPSHAVVAR, one of the signature trypsin cleavage peptides of lactoferrin, was strongly detected in our previous mass spectrometry analysis (data not published) and, therefore, was used in this study. The transition list for this target peptide was generated by the Skyline version 0.5 program (MacCoss Lab, University of Washington). The peptide sequence was pasted to the program, and the isotope modification was selected in the peptide setting option (Table 1).

Serial dilutions of the light peptide of 50, 150, 200, 300, and 600 fmol/μl were generated by diluting in 0.1% FA in the presence of 150 fmol/μl of heavy peptide. These mixed light and heavy peptides served as standards to construct the calibration curve.

Absolute quantitation of lactoferrin using MRM

All of the individual tear samples that were previously prepared for MRM were reconstituted using 20 μl of 150 fmol heavy peptide. Here, 1 μl of each prepared peptide standard and tear sample was injected into a 4000 QTRAP hybrid triple quadrupole linear ion trap mass spectrometer (Applied Biosystems/MDS Sciex) interfaced with a nanospray ion source and operating in positive ion mode, as published previously [20]. Briefly, all of the injected samples were concentrated and desalted onto a micro C18 precolumn (500 μm × 2 mm, Michrom Bioresources, USA) with H_2O/CH_3CN (98:2, 0.05% [v/v] TFA) at 15 μl/min. After 4 min, washing the precolumn was automatically switched (Valco 10-port valve, Houston, TX, USA) into line with a fritless nano column manufactured according to Gatlin and coworkers [21]. Peptides were eluted using a linear gradient of H_2O/CH_3CN (98:2, 0.1% [v/v] FA) to H_2O/CH_3CN (36:64, 0.1% [v/v] FA) at approximately 300 nl/min over 30 min. The precolumn was connected via a fused silica capillary (25 cm and 25 μm) to a low-volume tee (Upchurch Scientific, USA) and introduced into the 4000 QTRAP mass spectrometer. Samples were analyzed with an ion spray voltage of 2.4 kV, curtain gas flow of 12, and nebulizing gas flow of 5. For MRM analysis, quadrupoles were operated in a low resolution and the dwell time was 50 ms. The files