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Proteomic analysis of secretion from human transplanted submandibular gland replacing lacrimal gland with severe keratoconjunctivitis sicca

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ABSTRACT

Purpose: Proteomic analysis of secretions from transplanted or non-transplanted submandibular glands in patients with severe keratoconjunctivitis sicca and tears from normal eyes. *Experimental design*: Secretions from submandibular glands transplanted to replace lacrimal glands and non-transplanted submandibular glands were collected at 1 year from 5 patients with severe keratoconjunctivitis sicca undergoing transplantation, and tears were collected from 3 normal subjects. 2-D electrophoresis (2-DE), then mass spectrometry was used to identify proteins. Western blot analysis was used to confirm protein expression. *Results*: We identified 34 and 11 distinct proteins in the saliva from transplanted submandibular glands and tears, respectively. The saliva from transplanted submandibular glands contained almost all the proteins abundant in tear fluid. The functions of identified proteins in the saliva from transplanted submandibular gland were mainly immune response and anti-bacterial. In total, 7 proteins showed differential expression between the saliva of transplanted and non-transplanted submandibular glands. The upregulation of short palate, lung and nasal epithelium carcinoma-associated protein 2 and carbonic anhydrase VI was confirmed by Western blot analysis. *Conclusions*: Identified proteins in saliva from transplanted submandibular glands may protect ocular structures. These findings can help in understanding the functional status of transplanted submandibular glands.

1. Introduction

Keratoconjunctivitis sicca (KCS), also known as dry eye syndrome, is a common ophthalmological condition. The incidence is between 15% and 28% in people older than 60 years [1,2]. In China, about 30 million people have the disorder [3]. Treatments include the use of tear substitutes and surgical corrections such as obliteration of the lacrimal drainage pathways, which are effective only for mild cases but not for severe cases [3–5].

In 1986, Murube-del-Castillo first described microvascular autologous submandibular gland transplantation for surgical correcting severe keratoconjunctivitis sicca. The whole submandibular gland is transferred to the temporal region of the skull. The related blood vessels are anastomosed to the superficial temporal artery and vein. Wharton's duct is transplanted to the upper lateral conjunctiva fornix without connecting to the duct of the lacrimal gland. The gland is left denervated, and the damaged lacrimal gland is left *in situ* [4]. The procedure is now in use in several medical institutions in Australia, Germany, China, United Kingdom, and the United States [3,5–8]. Long-term follow-up revealed that secretion from the transplanted gland significantly improves dry eyes in both subjective and objective parameters [8].

However, whether the protein composition of the secretion from the transplanted submandibular gland differs from that of eye fluid or saliva of normal submandibular gland remains unknown. Previous studies showed that newly created tear film of KCS patients undergoing transplantation of submandibular gland remains mainly salivary, with lower osmolality relative to tears [8,9]. Therefore, proteomic examination of the composition of secretions from transplanted submandibular glands is important to fully understand the status of transplanted submandibular glands and further improves transplantation therapy for severe KCS.

Proteomics is a fast-growing discipline that has recently been used for analysis of whole saliva and parotid and submandibular-sublingual

Abbreviations: KCS, Keratoconjunctivitis sicca; 2-D, Two dimensional; SPLUNC2, Short palate, lung and nasal epithelium carcinoma-associated protein 2; CA VI, Carbonic anhydrase VI; CBB, Coomassie brilliant blue; WS, whole saliva; MS, Mass spectrometry; FA, formic acid

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glandular secretions, and proved to be a valuable approach in the field of saliva research [10–12].

In the present study, we performed proteomic analysis of secretions from transplanted or non-transplanted submandibular glands of patients with severe KCS and normal tears.

2. Materials and methods

2.1. Human subjects

The project was approved by the Scientific and Ethics Committee of Peking University. We included 5 patients with severe KCS (3 males, mean age 49.4 ± 12.5 years) with autologous submandibular gland transplantation on one side in the Department of Oral and Maxillofacial Surgery, Peking University School of Stomatology. We transplanted the submandibular gland as described [3] with modification, and the 5 patients underwent partial glandular transplantation (about 2/3 submandibular gland transplanted) because the secretion of 1/2 normal submandibular gland is enough to lubricate the ocular surface. In addition, all patients underwent surgery to reduce the size of the transplanted gland 1 year after transplantation because of epiphora or persisting excessive secretion. The transplanted submandibular gland was left denervated. All patients underwent complete systemic examination before transplantation. None had Sjögren's syndrome, symptoms of xerostomia or history of smoking. The underlying causes of the severe KCS were idiopathic (4 patients) and chronic keratoconjunctivitis (1 patient). Schirmer test results were <1 mm for all 5 patients, and scientigraphy with ^{99m}Tc pertechnetate revealed no hypofunction of submandibular glands before transplantation.

Three normal subjects (2 males, mean age 25.3 ± 1.9 years) were recruited for tear analysis. All subjects did not wear contact lens and had normal ocular and general health. None was taking any medication. All subjects gave their signed informed consent to be in the study.

2.2. Chemicals and reagents

Iodoacetamide (IAA), DL-dithiothreitol (DTT), urea, thiourea, glycerol, acetonitrile (ACN), trifluoroacetic acid (TFA) and 3-[(3-cholamidopropyl) dimethylammonio] propanesulfonate (CHAPS) were from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal antibody targeting the C-terminal peptide of human SPLUNC2 (VDNPQHKTQLQTLI) was raised from rabbits by Cowin Biotech Co. (Beijing, China). Anti-CA VI goat monoclonal antibody (targeting human CA VI) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.3. Sample collection and processing

2.3.1. Secretion samples

Unstimulated submandibular saliva was collected 12 months after the transplantation from the submandibular gland on the contralateral side to the transplantation side using the special collecting system as described by Wolff et al. [13]. The unstimulated secretion from the transplanted submandibular gland was collected 12 months after the transplantation from the inferior marginal tear strip of the impaired eye with a calibrated capillary tube. We collected at least 1 mL saliva from the non-transplanted or transplanted submandibular gland from each patient. Total collection time ranged from 25 to 35 min.

Tear fluid from normal subjects was stimulated by the yawn reflex elicited voluntarily by the subjects. The subject's lower eyelid was gently pulled down and the tip of a 20-μL sterilized glass microcapillary tube (Microcaps, Drummond Scientific, USA) was placed in contact with the tear meniscus near the lateral canthus. We collected about 500 μL tears from one eye of each subject. Total collection time ranged from 25 to 40 min.

Immediately after collection, 1/10 volume protease cocktail inhibitor (Sigma, USA) was added to the secretion samples to prevent proteolytic degradation. To remove debris, samples were centrifuged at 14,000 *g* for 15 min at 4 °C. Proteins in the samples were precipitated by use of the Liquor Protein Extraction Kit (Applygen Technologies, Inc., China). After centrifugation at 14,000 *g* for 15 min at 4 °C, the protein pellets were washed 3 times with ethanol, air dried, and solubilized in the buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, and 0.2% bio-lytes ampholyte pH 3–10 (rehydration solution). Protein concentration was measured by the Bradford assay (Bio Rad). Protein solutions were stored at −80 °C until use.

2.3.2. Tissue samples

Since all five patients underwent two operations, the first was partial glandular transplantation of submandibular gland, and the second was reducing the size of the transplanted gland 1 year after transplantation, tissues of normal submandibular glands were collected at the first operation while tissues from transplanted submandibular glands were collected at the second surgery. All the tissue specimens were snap frozen in liquid nitrogen for further procedure.

Tissue samples (0.1 g) were cut into pieces of about $2\times2\times2$ mm^3 and homogenized by use of homogenizer (Ultra-Turrax T10, IKA Lab Technology, Staufen, Germany) in an ice-cold denaturing lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin), then sonicated by use of a Sonifer II 450 (Branson, Danbury, CT) in an ice bath for 8 cycles, each consisting of 5-s sonication followed by a 10-s break, then held for 30 min on ice with periodic vortexing. The lysates were centrifuged at 14,000 *g* for 30 min at 4 °C. The protein concentration in the supernatants was determined by use of a Bio-Rad protein quantitation kit. Protein samples were aliquoted and stored at −80 °C for Western blot analysis.

2.4. Proteomic analysis

2.4.1. 2-D electrophoresis (2-DE)

Immobilized pH gradient (IPG) strips (17 cm, pH 3–10 nonlinear; Bio-Rad) were passively rehydrated for 1 h with 300 μL protein solution (150 μg for analytical gels and 1500 μg for preparative gels, respectively), covered with mineral oil and transferred to an isoelectric focusing (IEF) cell (Bio-Rad). IEF was performed with constant power (50 μA/IPG strip) at 50 V for 12 h of rehydration; 250 V for 30 min, linear; 1000 V for 1 h, rapid; linear ramping to 10,000 V for 5 h; and finally 10,000 V for 6 h.

The IPG strips were equilibrated with a buffer containing 50 mM Tris/HCl (pH 8.8), 6 M urea, 2% SDS, 20% glycerol, and 10 mM DTT for 15 min, washed with wash buffer (50 mM Tris/HCl, pH 8.8, 6 M urea, 2% SDS, 20% glycerol, and 200 mM iodoacetamide) for 15 min, transferred to 12% SDS-PAGE gels (18.5×20 cm, manually poured) and separated at constant current (10 mA for the initial 40 min and then 30 mA to the end). 2-DE was performed at least twice for each sample.

2.4.2. ESI-Q-TOF MS/MS and protein identification

The peptides from the tryptic digestion were analyzed with use of fused silica tubing (75 μm × 100 mm) packed with symmetry 300 C_{18}, 3.5-μm spherical particles with pore diameter 100 Å (Waters) on a Waters Capillary liquid chromatography system including 3 pumps A, B and C (Waters). Samples were injected at a flow rate of 20 μL/min with pump C and salts were removed on the precolumn (0.35×5 mm) packed with symmetry 300 C_{18}, 3.5-μm spherical particles with pore diameter 100 Å (Waters). The precolumn was connected in the 10-port switching valve and switched to the analytical column after the sample was desalted. Mobile phase A consisted of water/ACN (95/5, v/v) with 0.1% aqueous formic acid (FA). Mobile phase B consisted of water/ACN (5/95, v/v) with 0.1% FA. The separation was