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Proteome profiling of wild type and lumican-deficient mouse corneas

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ABSTRACT

To elucidate how the deficiency of a major corneal proteoglycan, lumican, affects corneal homeostasis, we used mass spectrometry to derive the proteome profile of the lumican-deficient and the heterozygous mouse corneas and compared these to the wild type corneal proteome. 2108 proteins were quantified in the mouse cornea. Selected proteins and transcripts were investigated by Western blot and quantitative RT-PCR, respectively. We observed major changes in the composition of the stromal extracellular matrix (ECM) proteins in the lumican-deficient mice. Lumican deficiency altered cellular proteins in the stroma and the corneal epithelium. The ECM changes included increases in fibril forming collagen type I, Collagen type VI, fibromodulin, perlecan, laminin β_2, collagen type IV, nidogen/entactin and anchoring collagen type VII in the $Lum^{+/-}$ and the $Lum^{-/-}$ mouse corneas, while the stromal proteoglycans decorin, biglycan and keratocan were decreased in the $Lum^{-/-}$ corneas. Cellular protein changes included increases in alcohol dehydrogenase, superoxide dismutase and decreases in epithelial cytokeratins 8 and 14. We also detected proteins that are novel to the cornea. The proteomes will provide an insight into the lumican-deficient corneal phenotype of stromal thinning and loss of transparency and a better understanding of pathogenic changes in corneal and ocular dystrophies.

1. Introduction

The healthy cornea is an avascular connective tissue-rich barrier of the eye that is both transparent and refractive for normal vision. The stroma, underlying the stratified epithelium, is a key regulator of corneal transparency and refraction, and it is comprised of specialized mesenchymal cells, the keratocytes and a collagen-rich extracellular matrix (ECM) they

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produce [1]. Injury, infection and corneal diseases can alter the exquisitely balanced cellular and the ECM content of the cornea and compromise its transparency, refractive power and barrier properties. The primary ECM constituents of the corneal stroma are fibrillar collagen types I, III and V and the small leucine-rich repeat proteoglycans (SLRPs), lumican, decorin, biglycan, keratocan and osteoglycin/mimecan [1]. Another SLRP, fibromodulin, is present in the developing cornea but restricted to the corneal periphery at maturity [2]. While the major protein components of the cornea have been identified, little is known of the corneal proteome, or how it is regulated by the major ECM components. The proteomics field has made significant advances in quantitative protein profiling by using isobaric tags for relative and absolute quantification (iTRAQ) that allow simultaneous analysis of four (iTRAQ 4 plex) or eight (iTRAQ 8 plex) different samples [3]. Thus, recent studies of the ocular surface in health and disease are beginning to incorporate these proteomic approaches [4,5].

Lumican is present in the interstitial ECM of the cornea, skin, intestinal submucosa, cartilage and bone [6]. Lumican is normally expressed by the mesenchymal fibroblasts, and transiently by the injured epithelia [7,8]. *In vitro* collagen fibrillogenesis assays and subsequent *in vivo* studies show that lumican and the other SLRP members of the cornea bind collagen and regulate collagen fibril growth [9–12]. The lumican-deficient ($Lum^{-/-}$) mice have cloudy corneas that are 40% thinner than those of the wild type mouse [9,13]. Our previous studies showed that the loss of corneal transparency in the $Lum^{-/-}$ mice was linked to abnormal collagen fibril architecture and increased light scattering [11,14,15]. We further found that wound healing in the $Lum^{-/-}$ corneas was delayed, and the stromal cells showed reduced apoptosis and increased proliferation, suggesting lumican-deficiency to have a broad pleiotropic effect in the cornea [16,17]. By contrast, deficiencies of the other corneal SLRPs, decorin [18], biglycan [19], fibromodulin [2,20] and keratocan [21] present a milder corneal phenotype. Therefore, we selected the lumican-null corneas for an in-depth proteomic analysis, and compared the corneal proteomes of lumican-expressing ($Lum^{+/+}$, $Lum^{+/-}$) and lumican-deficient ($Lum^{-/-}$) mice. We performed a multiplexed relative quantification of proteins by LC–MS/MS mass spectrometry using three different iTRAQ tags for $Lum^{+/+}$, $Lum^{+/-}$ and $Lum^{-/-}$ corneal protein extracts. About 2108 proteins were identified and quantified in the corneal extracts. These included several known cellular proteins, extracellular matrix collagens and proteoglycans, as well as proteins not previously linked to the cornea. Absence of one or both *Lum* alleles was associated with increases in several oxidative stress-related proteins, increased collagen type I, VI and decreases in the lumican-related SLRPs, decorin, biglycan and keratocan.

2. Materials and methods

2.1. Materials

The enzyme chondroitinase ABC was purchased from Associates of CAPE Cod incorporated. TCEP (Tris (2-carboxyethyl) phosphine), cysteine blocking agent (methyl methanethiosulfonate) and SYBR Green master mix were purchased from Applied Biosystems. Sequencing grade trypsin was from Promega. The oligonucleotide primers for PCR analyses were purchased from Eurofins Mwg Operon. KH_2PO_4, acetonitrile, KCl, formic acid, guanidine-HCl, sodium acetate, Tris–HCl, trifluoroacetic acid (TFA) and NaCl were from Sigma. TRIZOL and SuperScript III first-strand synthesis kits were from Invitrogen.

Rabbit anti-decorin (LF113) and anti-biglycan (LF159) were kindly provided by Dr. L. Fisher (NIH-NICDR). The following antibodies and reagents were purchased from Santa Cruz Biotech: goat anti-rabbit and anti-goat IgG-perixodase, and antibodies against β-Actin, Krt8, IGFBP2. The anti-Adh1 was obtained from Cell Signaling and Aldh1a1 from Abcam. The chemiluminescent HRP antibody detection reagent was obtained from Denville Scientific Inc. The source of the anti-collagen antibodies were as follows: Col6a1 from Dr. Monli Chu (Thomas Jefferson University), Col12 and Col14 were kindly provided by Dr. Manuel Koch (University of Cologne).

2.2. Protein sample preparation for mass spectrometry

$Lum^{+/+}$, $Lum^{+/-}$ and $Lum^{-/-}$ mice were generated as we described before [9]. All animals were housed in a specific pathogen-free mouse facility at Johns Hopkins University, according to protocols approved by the Animal Care and Use Committee. For the proteomics study, eight corneas per genotype were harvested from 8 week-old mice , rinsed in ice-cold PBS with protease inhibitor, and then frozen in liquid nitrogen (2.0 ml Biomasher, USA Scientific). After adding 150 μl of 0.5% SDS, the samples were centrifuged at 15,000 *g* for 30 s, sonicated for 1 min and centrifuged again at 15,000 *g* for 10 min. Since many of the commercially available cocktail protease inhibitors contain free amino groups, which compete for labeling with iTRAQ reagents, we did not include any protease inhibitors in the lysis buffer. Protease activity was minimized by lysing the samples directly in 0.5% SDS and subjecting the samples to ultrasonication, which is expected to cause denaturation and solubilization of proteins. Moreover, the samples were maintained on ice until the addition of trypsin to minimize any residual endogenous protease activity. The protein concentration in the supernatant was measured using the Bradford assay kit (Bio-Rad) and further confirmed by SDS-PAGE.

2.3. ITRAQ labeling

The $Lum^{+/+}$, $Lum^{+/-}$ and $Lum^{-/-}$ mouse corneal protein samples were differentially labeled using the iTRAQ reagent from Applied Biosystems (Fig. 1). Briefly, 70 μg of protein was treated with 2 μl of reducing agent TCEP at 60 °C for 30 min; and alkylated with 1 μl of cysteine blocking agent at room temperature for 10 min. Subsequently, the samples were diluted in iTRAQ dissolution buffer to reach a final SDS concentration of 0.025%. The protein samples were digested with 5 μg of sequencing grade trypsin for 12 h at 37 °C. The $Lum^{+/+}$, $Lum^{+/-}$ and $Lum^{-/-}$ digested samples, in a final volume of 35 μl were labeled with the iTRAQ reagents 114, 115 and 116 respectively, in 70 μl of ethanol for 2 h at room temperature, and the reactions were terminated by adding 100 μl water. The dried samples were reconstituted in a strong cation exchange