Experimental Eye Research 154 (2017) 53–63

Contents lists available at ScienceDirect

Experimental Eye Research

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

Research article

Anatomical differences of the protein profile in the rabbit sclera during growth

Ying Liu [a, c], Pingbo Zhang [a], Jianbo Pan [a], Mark Augustine D'Souza [a], Craig P. Dufresne [d], Richard D. Semba [a], Jiang Qian [a], Deepak P. Edward [a, b, *]

[a] Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, USA
[b] King Khaled Eye Specialist Hospital, Riyadh, Saudi Arabia
[c] Aier Eye Hospital, Changsha, Hunan, China
[d] Thermo Scientific, West Palm Beach, FL, USA

ARTICLE INFO

Article history:
Received 12 March 2016
Received in revised form
7 October 2016
Accepted in revised form 1 November 2016
Available online 5 November 2016

Keywords:
Sclera
Proteomics
LC-MS/MS
Differential expression
Age
Anatomic region

ABSTRACT

We aimed to investigate the proteome changes in anatomical regions of sclera during growth and development of the rabbit. Sclera from New Zealand white rabbits of three ages (1 month, 2 months and 6 months) was dissected into three segments - anterior, equatorial, and posterior. A total of 36 samples were divided into groups by age and anatomical region. Tryptic digests of total proteins were analyzed by liquid chromatography followed by tandem mass spectrometry (LC-MS/MS). Label-free quantification based on spectral counts was used to determine the relative protein abundance and identify proteins with statistically significant differences between age groups or anatomical regions of the sclera. Western blotting was performed to validate some of the differentially expressed proteins. A total of 840 non-redundant proteins was identified in the sclera at different ages and regions with protein and peptide false discovery rate (FDR) at $\leq$1.0% and $\leq$0.1%, respectively. Differentially expressed proteins were identified by comparing age or anatomical region. Among these, periostin showed decreasing abundance with age, while myocilin, latent-transforming growth factor beta-binding protein 2, hyaluronan, proteoglycan link protein 1 and selenbp1 showed increasing abundance with age. In mature rabbits, alcohol dehydrogenase showed region-related differences in the sclera. Periostin showed an age-related decrease while selenbp1 showed an age-related increase in abundance in the anterior region. Vitronectin and extracellular superoxide dismutase had greater expression with age in the equatorial and posterior regions, respectively. The age related differential expression of periostin and selenbp1 was confirmed by western blotting. In conclusion, the protein profile of sclera showed age- and region-related differences. The differential protein profiles provide a baseline for understanding changes in the protein expression in the young and mature rabbit that appears to show regional changes. The changes observed in the present study add to the existing knowledge about regional alterations in biomechanical properties of sclera during growth.

1. Introduction

The sclera forms a large proportion of the outer coat of the eyeball and serves as a supporting structure for the optic apparatus of the eye. In humans and other eutherian mammals, the sclera is predominantly composed of dense connective tissue interwoven into layers or lamellae, containing type I collagen along with lower amounts of type III and type V collagen, elastin, proteoglycans, and other structural proteins (Frost and Norton, 2007; Gentle and McBrien, 1999, 2002; McBrien and Gentle, 2003; McBrien et al., 2000; Norton and Rada, 1995; Rada et al., 2006). Fibroblasts that secrete extracellular matrix (ECM) also reside in the sclera. Scleral ECM plays a major role in the maintenance of its rigidity, strength and elasticity. In recent years, there has been an increasing interest in the molecular components of the sclera, particularly in relation to axial length changes. Many ECM proteins have been identified by cDNA-microarray technology or in situ hybridization or immunohistochemistry (Young et al., 2003, 2004). However, gene

* Corresponding author. PO box 7191. King Khaled Eye Specialist Hospital Riyadh 11642, Saudi Arabia.
E-mail address: dedwar39@jhmi.edu (D.P. Edward).

http://dx.doi.org/10.1016/j.exer.2016.11.001
0014-4835/

expression does not necessarily guarantee the presence of the protein, and genetic techniques are unlikely to predict the presence of the entire protein profile in a tissue. Therefore, a proteomics global approach such as LC/MS-MS becomes ideal to detect an entire profile of proteins and provide a relatively quantitative comparison among these proteins (Lu et al., 2007). The protein profiles provide useful information to characterize protein function and tissue physiology. Protein profiling of the scleral wall is critical to understanding the mechanisms that regulate ocular size and shape, as well as the etiology of abnormal eye expansion, myopia and inflammatory diseases.

More than a static outer collagenous ocular coat, the sclera is now known to undergo constant remodeling throughout life by dynamically altering its ECM composition and its biochemical and biomechanical properties in response to changes in the surrounding environment. These changes ultimately determine the shape and size of the globe (Rada et al., 2006). Scleral remodeling is a dynamic process that involves continual synthesis and degradation of ECM, such as degradation of collagen and elastic fibers (Rada et al., 2000, 2006). This process results in significant structural and functional changes in the sclera throughout life, therefore it is likely that the molecular composition of sclera is modified during the course of development, maturation and aging, and is responsible for some of the age-related changes. The age-related changes in scleral proteoglycan composition have been investigated in previous studies (Rada et al., 1997, 2000). It is known that physical function and biological behavior of the sclera depend largely on the interaction between its macromolecular components, so other components including proteins may also be similarly important and show some age related changes. However, there is little documentation on the global protein profile and age-related changes in sclera. Previous proteomic investigations using DIGE and 2DGE techniques, to investigate the changes in protein profiles of the sclera in animal myopia model (Frost and Norton, 2007, 2012; Zhou et al., 2010) have been limited in providing a comprehensive assessment of the scleral proteome.

In addition, the sclera also exhibits regional variation in structure, composition and function. First, the thickness of the human sclera is not uniform. Secondly, different scleral regions have specialized structures, for example, anterior sclera has the insertion of rectus muscle and the scleral spur serves as an attachment point for the trabecular meshwork while posterior sclera contains the scleral canal and the lamina cribrosa, in addition to sites where vessels and nerves enter the eye. Regional analysis of scleral glycosaminoglycans indicates that the sclera from different locations have variable amounts or types of proteoglycans (Trier et al., 1990). Fang Lu and colleagues used two-dimensional gel electrophoresis (2-DE) to analyze the protein profile of normal human scleral fibroblasts isolated from anterior and posterior sclera. They found that although the distribution and levels of expression in proteins are very similar between the anterior and posterior sclera in vitro, there were still approximately 4% of the proteins demonstrating at least a five-fold differential level of expression between the two regions (Lu et al., 2007). Functionally, the anterior sclera demonstrates a significantly higher modulus of elasticity than the posterior sclera (Rada et al., 2000). A better understanding of such regional variances may help explain the region-specific changes found in sclera related disease such as myopia and glaucoma. However, no research has been conducted that investigated the protein composition in different anatomical scleral regions.

To the best of our knowledge, a comprehensive analysis of the distribution of proteins in the sclera of human or animals is lacking in the literature. We aimed to establish normative protein profiles and investigate the differences between distinct anatomical regions of the sclera in rabbits at different growth stages using liquid chromatography-tandem mass spectrometry (LC-MS/MS). This study is intended to provide a basic understanding of scleral protein composition and facilitate the identification of alterations from normal expression that plays important roles in pathogenesis of diseases.

2. Materials and methods

2.1. Tissue procurement and sample grouping

Fresh enucleated eyes of New Zealand white (NZW) rabbits - young rabbits at age of one-month (YR-1M, n = 10), young rabbits at age of two-months (YR-2M, n = 10) and mature rabbits at age of 6 months (Macari and Machado, 1978) (MR, n = 10) were obtained from Pel-freeze Biologicals (Rogers, AR) within 6 h of euthanasia. Upon arrival, the eyes were dissected as follows: cornea was removed circumferentially along the limbus. The iris, ciliary body, lens, vitreous, retina, optic nerve and choroid were then removed completely to isolate the scleral tissue. All steps were done on a chilled plate to preserve the tissue's integrity. The inner wall of the sclera was scraped off to remove any adherent choroid. The sclera was then dissected into three anatomical regions: anterior sclera (AS, defined as a circumferential belt approximately 4 mm wide, as measured from the corneoscleral limbus), equatorial sclera (ES, a circumferential belt approximately 2 mm wide midway between the corneoscleral limbus and the optic nerve sheath), and posterior sclera (PS, sclera posterior to the equator, excluding the lamina cribrosa, and optic nerve and its sheath). Sclera from each region from at least 2 eyes at the same age was pooled as 1 sample. Sample pooling was necessary especially for the one month old rabbits since the rabbit eyes were small and various anatomic locations were sampled. Sclera was minced into small pieces with fine scissors and snap frozen in liquid nitrogen. The frozen scleral tissue was cryogenically ground into fine powder by using a freezer mill (Mixer Mill MM400, Retsch, Hann, Germany) and stored at −80 °C until use.

A total of 36 scleral samples were obtained and grouped by age into 3 major groups including YR-1M, YR-2M and MR, and each age group contained 3 subgroups by regions (AS, ES, PS). Similarly, samples were also categorized by region into 3 major groups: AS, ES and PS with each consisting of 3 subgroups by age (YR-1M, YR-2M and MR). 9 subgroups were formed for proteomics analysis with 4 samples in each.

No IACUC was required for this study since the eyes were obtained from a commercial source.

2.2. LC-MS/MS

2.2.1. Protein extraction and digestion

Protein extraction was performed according to the previously published technique with some modifications (Rada et al., 2000). Briefly each scleral powder sample was added to 3 ml of 4M guanidine HCL, 0.05 M NaOAc and 0.5 ml protease inhibitor cocktail (AEBSF, Aprotinin, Bestatin, EDTA, E-64, and Pepstatin [Sigma]) at pH5.8 and left overnight at 4 °C on a rotating mixer. Each sample was then centrifuged at 4000×*g* for 10 min at 4 °C. Supernatant was collected and centrifuged twice at 12,000×*g* for 10 min, also at 4 °C. The resultant supernatant was collected and the protein concentration was measured with Pierce 660 nm assay kit (Thermo Scientific) followed by acetone precipitation as follows: 150 μg of protein in 300 μl samples were added to 1.2 ml ice cold acetone and incubated at −70 °C for 30 min and then at −20 °C for 2 h. Samples were then centrifuged at 15,000×*g* for 15 min at 4 °C. After supernatant was removed, 1 ml of 80% acetone in water was added and allowed to stand at −20 °C for 1 h, and then centrifuged at