Neurobiology of Aging 34 (2013) 2659–2675

Contents lists available at SciVerse ScienceDirect

Neurobiology of Aging

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

Macula-less rat and macula-bearing monkey retinas exhibit common lifelong proteomic changes

Michael R.R. Böhm [a,b], Sonja Mertsch [a], Simone König [c], Tilmann Spieker [d], Solon Thanos [a,b,*]

[a] Institute for Experimental Ophthalmology, School of Medicine, Westfalian-Wilhelms-University Münster, Münster, Germany
[b] Interdisciplinary Centre for Clinical Research, Münster, Germany
[c] Integrated Functional Genomics, Interdisciplinary Centre for Clinical Research, Münster, Germany
[d] Gerhard-Domagk-Institute of Pathology, Münster, Germany

ARTICLE INFO

Article history:
Received 28 November 2012
Received in revised form 10 April 2013
Accepted 20 April 2013
Available online 30 May 2013

Keywords:
Aging retina
Proteomics
Protein expression
Mammals
Macula

ABSTRACT

The visual consequences of age-related alterations in the neural retina have been well documented, but little is known about their molecular bases. We performed a comparative proteomic analysis of the retinas in marmosets and rats to identify proteins for which the expression profiles are altered with maturation and aging. Protein profiles were compared in the newborn, juvenile, middle-age, and aged retinas using 2-dimensional gel electrophoresis. Matrix-assisted desorption ionization–time-of-flight mass spectrometry revealed common proteins in rats and marmosets that exhibited changes in concentration throughout life. Western blot, quantitative reverse-transcriptase polymerase chain reaction, and immunohistochemistry analyses of selected proteins and their mRNA were used to determine whether the changes identified by proteomics were verifiable at the cellular and molecular levels. We found 4 proteins common to both species (Parkinson disease [autosomal recessive, early onset] 7/DJ-1, stathmin, peroxiredoxin, and β-synuclein) whose concentrations were regulated with age. These findings were confirmed by Western blot, immunohistochemistry, and quantitative reverse-transcriptase polymerase chain reaction analyses. The proteins were localized in certain layers and subsets of cells within the retinas of both species. The expression of these proteins in the adult human retina was confirmed with immunohistochemistry. The present study is the first to provide evidence that the retina is physiologically characterized by specific lifelong changes in its proteome. These changes are independent of whether the retina bears a macula, occur in key functional pathways during the processing of visual signals, and might be involved in the development of age-related pathologic entities.

1. Introduction

Aging of the retina hampers visual function (Spear, 1993). However, the molecular mechanisms underlying physiological aging, which might be connected to pathologic alterations, such as age-related macular degeneration (AMD), are not well known. Various reasons exist for this discrepancy. First, the cellular diversity represented by the retina, which contains about 70 distinct functional cell subtypes (Masland, 2001), has made detailed molecular analyses difficult. Second, a great heterogeneity exists in the neuronal shapes and sizes that can mask alterations in specific types of neurons. Third, the 5 major neuronal types perform quite different functions, with the photoreceptors detecting light, the 3 types of interneuron (i.e., amacrine, bipolar, and horizontal) processing this visual input, and the retinal ganglion cells (RGCs) transmitting the information to the brain (see Sanes and Zipursky, 2010, for review). Finally, an additional major player, the retinal pigment epithelium (RPE) in the subretinal space, performs important functions in maintaining photoreceptor integrity and repair (Gu et al., 2012).

Visual function clearly declines in aged individuals, and some of the age-related changes in properties such as visual acuity (Gittings and Fozard, 1986; Weale, 1975) can be attributed to retinal changes (Jackson and Owsley, 2003; Jackson et al., 2002). Morphologically, the macula thickness decreases with age in normal eyes (Eriksson and Alm, 2009; Leung et al., 2012), which is consistent with the natural loss of cells in the retina (Katz and Robison, 1986; Kim et al., 1996). However, morphologic studies alone cannot unravel the underlying cellular mechanisms. In addition to minimal declines in the number of ganglion and photoreceptor cells, the number of dendritic arbors of RGCs decreases in the aged retina, explaining the changes that occur at the protein level (Samuel et al., 2011). The advent of proteomics has provided useful protocols for examining

* Corresponding author at: Institute for Experimental Ophthalmology, School of Medicine, Westfalian-Wilhelms-University Münster, Albert-Schweitzer-Campus 1, D15, Münster 48149, Germany. Tel.: +49-251-8356915; fax: +49-251-8356916.
E-mail address: solon@uni-muenster.de (S. Thanos).

0197-4580/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.neurobiolaging.2013.04.020

alterations in protein expression and discovering novel and common proteins when comparing different organisms and subsets of cells (Bonilha et al., 2004; Dörr et al., 2011). To this end, molecular-level proteomic approaches have revealed certain aspects of aging, in particular that of the rather homogeneous RPE cells, thereby providing a database for RPE aging that could facilitate our understanding of age-related diseases (Gu et al., 2012).

Understanding the cellular mechanisms of retinal aging will help determine the causes of age-related diseases such as AMD, the leading cause of vision loss and blindness (see Brucker, 2009; Nag and Wadhwa, 2012, for review). A critical issue associated with AMD is the central macular localization of the disease (Brucker, 2009). This prompted Ethen et al. (2006) to examine differences in the proteome in the central and peripheral parts of the retina with progression of AMD in humans. They found 26 proteins that exhibited changes at disease onset or with progression and at the end stage of the disease. By comparing the central and peripheral parts of the retina of adult monkey eyes (*Macaca fascicularis*), Okamoto et al. (2010) identified 5 proteins (arrestin-c, α-synuclein, epidermal fatty acid–binding protein, tropomyosin 1α chain, and heterogeneous nuclear ribonucleoproteins A2/B1) that were significantly more strongly expressed in the macula than in the peripheral retina. A2E is a key bisretinoid constituent of ocular lipofuscin that accumulates in retinal microglial cells and alters the physiology of microglia in pathologically relevant ways (Ma et al., 2013).

Some of the retinal proteins could be associated with macrophage activation and neoangiogenesis, as shown in mice (Jager and Klaver, 2007). Furthermore, cone function is severely affected in AMD, and cone degeneration has been demonstrated using opsin and synaptic markers in AMD-affected retinas (Shelley et al., 2009). Gouras et al. (2010) concluded that lipofuscin toxicity might not be the sole cause of AMD, thereby adding an additional line of evidence that AMD is multifactorial and complex. However, lipofuscin plays a prominent role, and both clinical studies and animal models have disclosed that AMD is characterized by multiple lesions, such as photoreceptor degeneration, abnormal RPE with increased lipofuscin, basal folding, and decreased melanosome degradation (Pennesi et al., 2012; Ramkumar et al., 2010). In addition, macula-specific increases in mitochondrial DNA damage, heteroplasmic mutagenesis, and diminished repair have been associated with aging and AMD severity (Lin et al., 2011). Moreover, certain genes were found to be expressed in 4 distinct clusters (macular, peripheral, young, or old retinas), including inhibitors of the *Wnt* signaling pathway, cyclin-dependent kinase 1, frizzled 10, and secreted frizzled-related protein 2 (Cai et al., 2012).

In the present study, we used 2-dimensional gel electrophoresis and subsequent liquid chromatography (LC)-mass spectrometry (MS)/MS, as well as LC-MS^E (elevated energy) to identify those proteins whose expression changes throughout the lives of rats and monkeys (*Callithrix jacchus*). We selected the proteins whose expression changed in both species and characterized them qualitatively and quantitatively using Western blot (WB), quantitative reverse-transcriptase (qRT) polymerase chain reaction PCR, and immunohistochemical (IHC) analyses. In addition, the staining pattern of these proteins was studied in human retinal sections. Knowledge of the comparative protein changes at the interspecies level will provide insight into the common biological processes associated with the physiological and pathologic changes in age-related vision.

2. Methods

2.1. Animals

All animal work was conducted under the guidelines of the Animal Welfare Act and under the oversight and approval of the University and Governmental Institutional Animal Care and Use Committee (permission numbers 84-02.04.2011.A132 for rats and 8.87-50.10.46.09.018 for monkeys). Human sections of a 22-year-old and 73-year-old retina were obtained after obtaining written consent from each donor in accordance with the permit issued by the Ethics Committee of the University for Anonymous Use of Histological Material for Research Purposes.

Sprague-Dawley rats were housed in standard animal rooms under a 12/12-hour light–dark cycle, with food and water provided ad libitum. A total of 75 rats were used that covered the following ages: postnatal day (P)0 (i.e., the day of birth), shortly after opening their eyes (P16), shortly after gaining lens transparency (P23), at a juvenile stage of life (P60), at a young adult stage of life (P90), at middle age (P360), and aged (P30 months). Marmoset (*C. jacchus*) eyes were obtained from cadavers after killing monkeys from the local monkey colony of the university. The experiments were performed on 26 retinas and 10 eyecups obtained from 18 male and female marmosets aged P0, P10, P8 weeks (P8w), P4 months (P4m), P22 months (P22m), and 8 years (P96 months). The eyes were placed on ice and were used immediately; for the proteomic, IHC, WB, and qRT-PCR analyses, the eyes were obtained 10 to 20 minutes after death. The eyecups were removed and placed in Hank's balanced salt solution, in which all subsequent preparation steps were conducted under sterile conditions. The retina was dissected and isolated, flat-mounted on a nitrocellulose filter, and separated from the vitreous with fine forceps. The isolated samples of the tissues were frozen in liquid nitrogen. For IHC, the eyecups were frozen in Tissue-Tek (Sakura Finetek Europe, Alphen an den Rijn, The Netherlands). The isolated samples were stored at −80 °C until being used further.

2.2. Two-dimensional polyacrylamide-gel electrophoresis and proteomics

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) (O'Farrell et al., 1977) was performed on retinal samples of various ages. Retina proteins were analyzed using 2D-PAGE followed by MS investigation of spots of interest and the application of a special MS detection scheme for the study of complex mixtures (MS^E, Waters Corp) to total protein digests. Retinal probes were boiled in 10% sodium dodecyl sulfate (SDS; Sigma-Aldrich, Taufkirchen, Germany) and homogenized in 2D-PAGE lysis buffer (7 M urea and 2 M thiourea; Merck, Darmstadt, Germany), 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propan sulfonate (USB, Cleveland, OH, USA), 40 mM Tris base (Carl Roth, Karlsruhe, Germany), 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich), and 10 mM dithiothreitol (Roche, Mannheim, Germany). The final SDS concentration was 0.25%. Soluble protein (200 μg, according to the Bradford test), together with 2% immobilized pH gradient buffer (pH 3–10; Amersham Biosciences Europe, Freiburg, Germany) and 20 mM dithiothreitol were loaded onto strips (pH 4–7, 18 cm; Immobiline DryStrip, Amersham Biosciences Europe) and rehydrated overnight. The rehydrated strips were focused on an electrophoresis unit (Multiphor II, Amersham Biosciences Europe) at approximately 80 kVh. Focused immobilized pH gradient strips were incubated twice for 15 minutes in equilibration solution (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% wt/vol SDS, and a trace of bromophenol blue; Merck), with 1% β-mercaptoethanol and 2.5% iodoacetoacetamide added to the first and second equilibration steps, respectively. For the second dimension, the equilibrated immobilized pH gradient strips were fixed with 0.5% wt/vol melted agarose (Merck) on homogeneous 12.5% SDS gels (Rotiphorese Gel 30, Carl Roth, Karlsruhe, Germany). The proteins were separated using vertical SDS-PAGE (BioRad, Munich, Germany). Protein spots, which were initially labeled with colloidal Coomassie