Age-related changes in the transcriptional profile of mouse RPE/choroid

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Ida, Hisashi, Sharon A. Boylan, Andrea L. Weigel, and Leonard M. Hjelmeland. Age-related changes in the transcriptional profile of mouse RPE/choroid. Physiol Genomics 15: 258–262, 2003. First published September 30, 2003; 10.1152/physiolgenomics.00126.2003.—To evaluate the age-related changes in gene expression occurring in the complex of retinal pigmented epithelium, Bruch’s membrane, and choroid (RPE/choroid), we examined the gene expression profiles of young adult (2 mo) and old (24 mo) male C57BL/6 mice. cDNA probe sets from individual animals were synthesized using total RNA isolated from the RPE/choroid of each animal. Probes were amplified using the Clontech SMART system, radioactively labeled, and hybridized to two different Clontech Atlas mouse cDNA arrays. From each age group, three independent triplicates were hybridized to the arrays. Statistical analyses were performed using the Significance Analysis of Microarrays program (SAM version 1.13; Stanford University). Selected array results were confirmed by semi-quantitative RT-PCR analysis. Of 2,340 genes represented on the arrays, ~60% were expressed in young and/or old mouse RPE/choroid. A moderate fraction (12%) of all expressed genes exhibited a statistically significant change in expression with age. Of these 150 genes, all but two, HMG14 and carboxypeptidase E, were upregulated with age. Many of these upregulated genes can be grouped into several broad functional categories: immune response, proteases and protease inhibitors, stress response, and neovascularization. RT-PCR results from six of six genes examined confirmed the differential change in expression with age of these genes. Our study provides likely candidate genes to further study their role in the development of age-related macular degeneration and other aging diseases affecting the RPE/choroid.

retinal pigmented epithelium; aging; cDNA microarray; choroid

AGING, A UNIVERSAL PHENOMENON in all eukaryotes, is associated with physiological changes and pathological conditions. The most prominent and least treatable of age-related pathologies of the eye is age-related macular degeneration (AMD). The histopathology of AMD exhibits abnormal changes in the retinal pigmented epithelium and choroid (RPE/choroid), and the loss of RPE cells is central to disease development (11, 20). The pathogenesis of AMD is complex, and disease progression is associated with environmental as well as genetic factors. Currently there are only two known epigenetic risk factors: age and smoking (25, 36). Identifying processes involved in modulating aging of the RPE/choroid may contribute to understanding the pathogenesis of AMD, as well as increase the understanding of aging changes in vivo in general.

Mice have been used as a model system for studying the association of pathological aging changes with the early stages of AMD. Senescence-accelerated mouse types and mice exposed to blue light and/or high-fat diets show changes in the RPE/choroid that are similar to changes seen in the development of AMD in humans (7, 10, 21). Therefore, we have made an initial study of changes in the transcriptome of the mouse RPE/choroid as a function of age. We utilized the C57BL/6 mouse, since this strain was also used in previous models examining the influence of diet and blue light exposure on age-related changes in the RPE/choroid. Microarray analysis was performed on sets of animals at 2 and 24 mo of age by means of a commercial array system including 2,340 genes. Statistical analysis of our data identified 150 genes that were regulated by age. This set of differentially expressed genes will serve as candidates for further study into aging in general, and AMD in particular.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice were obtained from the National Institute on Aging (NIA). After arrival from NIA, the mice were housed in cages for 2 wk to let them adjust to their new environment. At ~2 or 24 mo of age, the mice were killed by cervical dislocation. All procedures and protocols were approved by the UC Davis Animal Use and Care Advisory Committee.

RNA isolation. Globes were removed from each mouse and placed immediately into a petri dish kept on ice and containing PBS-EDTA buffer. Any extraneous tissue was removed by gently washing the eye with cold PBS-EDTA buffer. After transfer of the eye to a clean petri dish containing ice-cold buffer, it was dissected in an RNase-free environment using a stereo-zoom microscope (model SMZ800; Nikon, Tokyo, Japan). The anterior segments were removed with a circumferential incision of the eye. Posterior segments were then separated from the whole retina. The pigmented layer including RPE/choroid was scraped gently from the sclera as a sheet in less than 50 μl of RNAlater buffer (Qiagen, Valencia, CA). The RPE/choroid from both eyes was placed in a tube containing 600 μl RLT lysis buffer (Qiagen). Before RNA isolation, tissues were first homogenized with a 22-gauge needle and a QIAshredder spin column. Total RNA was isolated using the Qiagen RNeasy kit (Qiagen) following the manufacturer’s protocol. As part of the RNA isolation proto-

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col, any DNA contamination was removed by incubation with DNase I. The yield of total RNA was determined using the RiboGreen RNA quantitation kit (Molecular Probes, Eugene, OR) and a spectrophotometer (model PC1; ISS, Champaign, IL) to measure the fluorescence of the RNA-bound dye (excitation wavelength of 485 nm, emission wavelength of 530 nm). The typical yield of total RNA per mouse was 0.5–1 µg (both eyes).

Preparation of cDNA probes. The Atlas SMART probe amplification kit (Clontech, Palo Alto, CA) was used for the preparation of double-stranded cDNA probes. First-strand cDNA synthesis was accomplished by adding 500 ng of total RNA, the 3′ SMART CDS primer II A, the SMART IIA oligonucleotide, and PowerScript reverse transcriptase to the reaction mixture (final volume of 50 µl). T4 gene 32 protein (Ambion, Austin, TX) was also added to the reaction, to enhance the yield of double-stranded cDNA during the PCR amplification step (3). Double-stranded cDNA (5 µl) were generated by exponential long-distance PCR amplification using the Advantage 2 polymerase mix and the 5′ Primer II A from Clontech. Starting from 500 ng of total RNA, each array contained cDNA synthesized in 2–5 µg of double-stranded cDNA. For each of the six mice studied, an amplified cDNA preparation was made.

The SMART cDNA probe labeling kit from Clontech was used to prepare [32P]-labeled cDNA. Each reaction contained 500 ng of purified cDNA, the CDS primer mix specific for the array to be probed (Clontech), Klenow enzyme (Invitrogen, Carlsbad, CA), and 50 µCi of [α-32P]dATP (3,000 Ci/mmol) (ICN Biomedicals, Costa Mesa, CA). Unincorporated nucleotide was removed using the Bio-Spin 30 column (Bio-Rad, Hercules, CA). Typically, 20–35 × 106 cpm of [α-32P]dATP was incorporated into 500 ng of probe.

Screening cDNA expression arrays. Labeled cDNA probes were denatured and hybridized to the Clontech Atlas nylon mouse cDNA arrays 1.2 I and 1.2 II (2,340 genes total). Both types of arrays were hybridized to each of the six mice studied (three 2-mo-old mice and three 24-mo-old mice) for a total of 12 arrays analyzed. Thus, for each age group, independent triplicates were performed. Array hybridization was done according to the manufacturer’s instructions, except that the hybridization and wash temperatures were 65°C. Arrays were exposed to a phosphor imaging screen for 3–5 days and scanned at 50-µm resolution in a PhosphorImager instrument (Storm 860; Molecular Dynamics, Sunnyvale, CA).

Image and statistical analysis. The gel images from the PhosphorImager were exported to the AtlasImage 1.5 software (30) and were synthesized by MWG Biotech (High Point, NC). RT-PCR reactions were conducted using RNA from animals subjected to microarray analysis. The resulting PCR products were visualized by ethidium bromide-stained agarose gels (Bio-Rad). Band intensities were quantitated using the FluorChem 8800 Imaging System (AlphaInnotech, San Leandro, CA). Gene-specific band intensities were normalized to the intensity of the 18S PCR product that had been amplified in the same reaction. The ratio of gene-specific product of 24-mo-old mouse sample to 2-mo-old mouse sample was determined. Each sample was performed in triplicate, and the ratios were averaged to obtain the mean and SE values for each gene. A ratio was considered significant if the P value from a paired two-sample for means f-test was less than or equal to 0.05.

RESULTS

Preparation of cDNA Probes from the RPE/choroid. To show changes in the RPE phenotype with age, we utilized microarray analysis to identify global changes in the mRNA expression pattern in male mice at the ages of 2 and 24 mo. Two sets of three male C57BL/6 mice were killed, the eyes were dissected on ice, and total RNA was isolated from the RPE/choroid under RNase-free conditions.

Amplifications of first-strand cDNA from samples of 2- and 24-mo-old mice were performed using the SMART amplification kit (Clontech) as described in MATERIALS AND METHODS. The exponential phase of amplification was determined by analyzing the gel electrophoresis pattern of the PCR products generated for different numbers of cycles. The resulting cDNA preparations appeared on an agarose gel as a strong smear from 0.5 to 6 kbp, with some distinct bands (data not shown). The reproducibility of amplified cDNA preparations was confirmed by microarray analysis of independent amplifications from the same RNA source. From a scatter plot of pair-wise comparison, a correlation factor (reported as an $R^2$ value) of 0.96 was obtained (data not shown). This compares favorably with similar studies done with RNA samples amplified using the T7 RNA polymerase protocol, which reported $R^2$ values ranging from 0.92 to 0.98 (19, 34).
Statistical analysis of microarray data. The SAM program (version 1.13; Stanford University) was used to identify significant differential gene expression using a two-class unpaired response analysis. SAM calculates a significance score for each gene based on the gene expression change relative to the standard deviation of repeated values. Genes with a score greater than an adjustable threshold are considered potentially significant. From the threshold value chosen by the user, SAM calculates the false discovery rate (FDR), which is defined as the number of false positives divided by the number of potentially significant genes. An FDR of 7.2% was chosen so that a significant number of genes could be examined without a high probability of finding many false positives. A fold change cutoff was not used in determining differential gene expression because the statistical analysis described above was used.

Number and classification of genes altered with age. Of the 2,340 genes represented on the two arrays, we found a total of 1,404 or ~60% that were expressed. Of these, a total of 150 genes were differentially expressed at an FDR of less than or equal to 7.2% (see Supplemental Table 1, available at the Physiological Genomics web site). This represents ~12% of the total number of genes expressed. Nearly all of the differentially expressed genes (148 genes) were upregulated in the 24-mo-old mice. Supplemental Table 1 shows that all functional categories are represented by these differentially expressed genes, with signal transduction and proteases including the most number of genes. With aging, we found an increase in expression of genes that are involved in the immune response (17 genes), neovascularization (6 genes) and in stress responses such as oxidative stress (11 genes), DNA damage (4 genes), and heat shock (3 genes). Also upregulated with age are genes encoding lysosomal proteases and inhibitors (10 genes), many of which are also induced during the stress responses. The two genes found to be downregulated with age encode carboxypeptidase E and the chromatin protein HMG14.

Confirmation of differential gene expression by RT-PCR. Semi-quantitative RT-PCR was performed for six of the gene products that the array results indicated to be differentially expressed. For our survey, we chose genes whose FDR varied from 1.3 to 7.2%. These genes came from various functional groups and represented genes from both array types used in this study. For each gene product measured, PCR amplification was terminated during the exponential phase of the reaction, and the ratios were normalized using the 18S product. Statistical analysis of RT-PCR reactions confirmed that all six genes are statistically significantly upregulated with age (Fig. 1). Gene expression ratios for five of the genes correlated well between the array analysis and the RT-PCR experiment, regardless of the FDR value of the gene.

DISCUSSION

To find candidate genes for the pathological aging changes of the RPE/choroid, we performed microarray analysis to discover age-related changes in gene expression in the RPE/choroid of the C57BL/6 mouse. Using a commercial nylon membrane cDNA array system, we found that 150 genes were differentially expressed between mice 2 and 24 mo of age. These represented ~12% of all genes expressed. From the genes upregulated with aging, we found many that fell into six functional categories: immune response, proteases and protease inhibitors, oxidative stress, DNA damage, heat shock, and neovascularization.

In our studies we used three individual animals from each age group (2 mo and 24 mo), one at a time for the arrays. To do this, we amplified individual RNA samples instead of pooling several samples for each array. Without RNA amplification, ~20–30 mice would have been needed per array. To determine significant differential gene expression with aging, data from the 2- and 24-mo-old replicates were compared with a two-class unpaired response analysis using the SAM program (33). Some studies use only a fold change cutoff to define significance (38), but the validity of this approach has been questioned. Tusher et al. (33) found Northern blot analysis showed little correlation with genes identified by fold change but a strong correlation

1 The Supplementary Material (Supplemental Table 1, a list of genes that are differentially expressed with age) for this article is available online at http://physiolgenomics.physiology.org/cgi/content/full/000126.2003/DC1.
with genes identified by SAM. With the FDR of 7.2% that we chose, we would expect ~1 of 14 genes randomly chosen from our significant genes list to be falsely significant. When we performed semi-quantitative RT-PCR for six of the genes identified by SAM, statistical significance in differential gene expression was confirmed (Fig. 1). Gene expression ratios obtained by RT-PCR for five of the six genes correlated well with the microarray results. However, for cathepsin S the ratio obtained from RT-PCR analysis was lower than that obtained by microarray. The large change in gene expression indicated by the array may be hard to duplicate by the RT-PCR method. The array technology itself may be nonlinear. Alternatively, it is possible that for the 24-mo-old mouse sample, the high expression of the cathepsin S gene may place the amplified product out of the linear range of the PCR amplification, even though the amplified product for the 2-mo-old mouse was at the lowest end of the linear range that is detectable by ethidium bromide staining.

Microarray analyses of age-related changes have been reported in a variety of mouse tissues (4, 5, 13, 16, 17). These studies show that the transcriptional response to aging is tissue specific, suggesting that tissues are subjected to different stresses during aging. An age-related increase in expression of inflammatory response and stress response genes was seen in the cerebellum, neocortex, liver, and heart of the mouse (4, 5, 17). However, aging did not induce inflammatory response genes in the hypothalamus, cortex, or skeletal muscle, and no stress response genes were induced in the hypothalamus or cortex (13, 16). This is the first microarray analysis to examine global changes in gene expression with age in the mouse RPE/choroid. Our findings indicate there is an age-related increase in expression of inflammatory response and stress response genes in the RPE/choroid. Compared with other mouse aging studies, our investigation found far fewer genes downregulated with age. There are several possible explanations for this. First, none of these studies involved the RPE/choroid, thus this low level of downregulation could be a tissue-specific response. Second, we may have missed some downregulated genes because they were not present on the commercial arrays we used, or they were not considered significantly downregulated because of their high FDR values. All of the genes reported to be downregulated with age in other mouse tissues are either not present on the arrays we used, or the FDR values for those genes are too high for the results to be considered significant. Based on our results, one cannot assume that the age-related transcriptional response of the RPE/choroid is predominantly an upregulation of gene expression. At 2,340 genes, these arrays represent less than 10% of the entire mouse genome.

Because of the relatively small number of genes present in our microarrays, this study is not comprehensive. However, even though the scope of our study is somewhat limited, our analysis did identify several candidate genes for retinal diseases. The cathepsins and matrix metalloproteinase 3 are involved in extracellular matrix remodeling (22, 27, 29, 35). Lysosomal enzymes are needed for the digestion by the RPE of spent photoreceptor outer segments (2, 37). Inhibition of cathepsin D and S activities leads to an accumulation of autofluorescent outer segments thought to be undigested photoreceptor segments similar to that seen in normal aging (12, 14). Transgenic mice with decreased cathepsin D activity and rats with downregulation of cathepsin S production show numerous retinal changes that occur in both normal aging and AMD (15, 28). Reduced proteinase activity involved in normal turnover of the extracellular matrix may affect the age-related thickening of Bruch’s membrane and the deposition of basal laminar deposits, which are precursors to AMD. Generation of reactive oxygen species (ROS) and lipid peroxides in the RPE is due in part to the phagocytosis of photoreceptor outer segments (18, 23, 32). Increased levels of lipid peroxidation in the RPE also occur with aging (8). The activity of GPX3 (plasma glutathione peroxidase 3, a secreted protein) against hydroperoxides, including lipid peroxides, indicates it may play an important role in protection against oxidative stress in the interstitial spaces surrounding the RPE especially during aging. Oxidative stress has been implicated in AMD, and various studies have found an association of decreased glutathione peroxidase activities with AMD (6, 24, 26).

Our present study is a starting point, providing candidate genes for further study of their possible role in the processes of aging and diseases of the retina. Ongoing studies in the laboratory include confirming both the candidate’s site of expression as well as relative age-related changes at these sites. Further microarray studies using a larger array system such as the Affymetrix MOE 430 GeneChip set and/or arrays containing eye-specific mouse genes will give a more comprehensive profile of mouse RPE/choroid gene expression (9).

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