Protein Analysis of Human Maculae in Relation to Age-Related Maculopathy

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BACKGROUND: Age-related maculopathy (ARM) is the most common cause of blindness in the elderly in the western world. Its early stage is characterized by many histopathologic changes, including two extracellular deposits, basal laminar deposit (BLD) and drusen. The origin and chemical composition of BLD and drusen are unknown and are considered to be important in the development of ARM, so we analyzed proteins in human macular tissue associated with ARM.

EXPERIMENTAL DESIGN: Homogenized macular extracts of 15 human eyes with ARM and 10 age-matched control eyes were examined by two-dimensional electrophoresis. The proteins in the gels were silver-stained, and the obtained protein patterns were analyzed by a computer-imaging system.

RESULTS: Five glycoproteins were specifically present in human maculae with ARM (p = 0.0009). One of the spots was characterized by sequence analysis as haptoglobin β-chain, and another had a high homology with a part of the interphotoreceptor retinoid-binding protein precursor. However, the 100% matching of the latter was not statistically significant because we could only sequence eight amino acids of this protein.

CONCLUSIONS: The known association between haptoglobin β-chain and atherosclerosis and the increase of this glycoprotein in human maculae with ARM supports the recently described relationship between atherosclerosis and ARM found in an epidemiologic study. Furthermore, the neovascular growth-stimulating properties of haptoglobin warrant further research into haptoglobin as a possible inducing agent of late stages of ARM.

Additional key words: Age-related macular degeneration, Subretinal neovascularization, Atherosclerosis, Drusen, Basal laminar deposit, Retina.

Age-related maculopathy (ARM) embraces many different histopathologic and morphometric changes in the central part of the human retina, such as the accumulation of lipofuscin in the retinal pigment epithelium (RPE), atrophy of the choriocapillaris, thickening and calcification of Bruch's membrane, drusen, and basal laminar deposit (BLD) (1–8). Drusen and BLD are both extracellular deposits, which are easily observed by light microscopy. The former are located within Bruch's membrane; the latter are located between Bruch's membrane and the RPE. The late stages of ARM, called age-related macular degeneration (AMD), appear in two forms: geographic atrophy and neovascular AMD. The RPE and consequently the photoreceptor cells disappear in geographic atrophy (5, 8–10). In neovascular or "exudative" AMD, new vessel ingrowth from the choriocapillaris into the subretinal space is observed (8, 9, 11, 12). Both forms of AMD can severely affect central vision and are the main cause of blindness in the elderly in the western society (13–15). The histopathology of this disease is well described, but the information on its pathogenesis is still incomplete (5, 8, 9, 11, 16).

Large drusen and BLD (and the chemical composition of these deposits) are assumed to be important determinants in the pathogenesis of AMD (2, 11, 17–21). It is known that drusen contain lipids (22–24) and probably also proteins (17). In a previous study, we proved that BLD contains carbohydrate structures most likely part of glycoproteins (25). To the best of our knowledge, there is no information on the protein components of age-related structures in human maculae. Therefore, we compared in this study the protein composition of ARM maculae with those of healthy (age-matched) human maculae.

EXPERIMENTAL DESIGN

Preparation of Human Macular Tissue

We obtained 25 human eye bank eyes from 16 subjects aged between 46 and 91 years of age (mean age 78 years); post mortem times were from 5 to 15 hours. From these eyes, 1 cm², with the macula in the middle, was cut out. This piece of tissue was horizontally divided through the fovea in the direction of the optic nerve head in two equal parts. From one half, the choroid and retina together were separated from the
sclera and vitreous and were subsequently frozen and stored in liquid nitrogen until further use. The other half was fixed in phosphate-buffered formaldehyde (4% v/v, pH 7.4, for 24 hours at room temperature). The formalin-fixed part was embedded in paraffin for histological classification (6). Serial paraffin sections (5 μm) were stained with hematoxylin and eosin, periodic acid-Schiff, and Mallory staining. The maculae were classified according to their histologic gradation of BLD (Class 0 to 3) and drusen (Class 0 to 3) (6). In brief, BLD Class 0, resembled no BLD; Class 1, small solitary patches of BLD; Class 2, a thin continuous layer of BLD; and Class 3, a thick layer of BLD of at least half the height of the RPE cells. Drusen Class 0, resembled no drusen; Class 1, 1 to 3 drusen; Class 2, 4 to 10 drusen; Class 3, many or confluent drusen (6). No great disturbances in the architecture of the maculae are present in maculae with both BLD Class 0 or Class 1 and drusen Class 0, so these eyes were categorized as control samples. All other maculae were classified as samples with ARM. The histologic classification of ARM maculae is consistent throughout a macula (6). Therefore, it was assumed that one-half of a macula is representative of a whole macula. There was no clinical data available on the donor tissues, so the classification was fully based on the histologic sections.

**TWO-DIMENSIONAL ELECTROPHORESIS**

The choroid and retina, frozen in liquid nitrogen, were defrosted and homogenized together. Two-dimensional electrophoresis of this material was performed, and the proteins in the gels were subsequently visualized with a silver staining technique. The M, and isoelectric point (pI) values of the proteins were calculated by a computer-imaging system. Finally, N-terminal amino acid sequences of the proteins of interest were determined.

**RESULTS AND DISCUSSION**

**RESULTS**

Fifteen eyes of nine subjects aged between 46 and 91 years (mean age 79 years, SD 13 years) were histologically classified as having ARM (Subjects 8 to 16, Tables 1 and 2). There were no late stages of ARM included in this study. Ten eyes of seven subjects aged between 62 and 83 years (mean age 77 years, SD 8 years) were classified as healthy (Subjects 1 to 7, Tables 1 and 2). The mean age of both groups was not significantly different (p = 0.74).

For comparison of the protein composition of ARM maculae and control maculae, we used only one eye of each subject (n = 16, Table 1). This was always the left eye in case we had used two eyes of one subject in our experiments.

There was a substantial amount of five proteins (P1 to P5) in human maculae with BLD (Class 2 and 3) and/or drusen (Fig. 1A, Table 1). These five proteins were scarce or absent in all control maculae (Fig. 1B, Table 1; p = 0.0009). We could not find any differences in the amount and the composition of the proteins between the left and right eye of one subject (n = 9, r = 0.74, p = 0.02). The amounts of protein P1 to P5 in the right maculae of these donors are listed in Table 2. The use of albumin (Fig. 1A) as an internal standard for blood content shows that the increase of the five proteins was not due to small differences in composition of the macular samples, for instance caused by varying amounts of blood in the choroid.

The five proteins were characterized by two-dimensional electrophoresis as follows: Protein spot 1 (P1) with M, 38900 and pI 6.25; protein spot 2 (P2) with M, 39200 and pI 6.14; protein spot 3 (P3) with M,
Table 3. Sequence Analysis of N-Terminal Amino Acids

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mₗ (×10⁶)</th>
<th>pI</th>
<th>Description</th>
<th>N-Terminal Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>38.9</td>
<td>6.3</td>
<td>?</td>
<td>—</td>
</tr>
<tr>
<td>P2</td>
<td>39.2</td>
<td>6.1</td>
<td>IRBP</td>
<td>PHRXPRAX</td>
</tr>
<tr>
<td>P3</td>
<td>40.2</td>
<td>6.0</td>
<td>Hp β-chain</td>
<td>ILGGHDLAKGxPPWQ</td>
</tr>
<tr>
<td>P4</td>
<td>41.8</td>
<td>5.8</td>
<td>Hp β-chain</td>
<td>—</td>
</tr>
<tr>
<td>P5</td>
<td>42.6</td>
<td>5.6</td>
<td>Hp β-chain</td>
<td>—</td>
</tr>
<tr>
<td>P6</td>
<td>43.8</td>
<td>6.4</td>
<td>Creatine-kinase PFxNxAHLKLR</td>
<td>FPAEDFDP</td>
</tr>
<tr>
<td>A</td>
<td>63.0</td>
<td>6.0</td>
<td>Albumin</td>
<td>—</td>
</tr>
</tbody>
</table>

* a Glycoproteins P1 to P5 were specifically present in human maculae with ARM. Protein P6 (creatine-kinase) was used as internal calibration marker for molecular weight, and protein A (albumin) was used as internal calibration marker for protein content. Hp, haptoglobin; x, nondeterminable amino acid (most likely serine).

* b Compared with Mₗ of molecular weight standards and isoelectric point (pI) of GAD3PDH carbamylates.

* c Concluded after comparison with plasma and RBC protein maps (26).

... and 3) or drusen or in maculae with both of these deposits.

With sequence analysis, we found 93% (14/15) identity in the first 15 N-terminal amino acids of P3 (Table 3) compared with the first 15 N-terminal amino acids of the β-chain of haptoglobin with a p value of 10⁻⁵.

Fig. 1. A. Two-dimensional electrophoresis of proteins in human macular tissue with age-related maculopathy. Note the presence of five protein spots (P1 to P5) that do not (or slightly) appear in healthy human maculae (B). Protein spot 6 (creatine-kinase, Table 3) and albumin (A, Table 3) were used as internal calibration markers. Macular tissue from Subject 12 (Table 1) was used for this figure. B. Two-dimensional electrophoresis of proteins in healthy human macular tissue. Proteins P1 to P5 are not expressed at all, whereas all the other protein spots are comparable with those in ARM maculae (A). Macular tissue from Subject 4 (Table 1) was used for this figure.

Fig. 2. Two-dimensional electrophoresis of proteins in human macular tissue with age-related maculopathy after treatment with N-glycanase. The same macular tissue was used as in Fig. 1A. N-glycanase removes the asparagine-linked carbohydrate side-chains from glycoproteins P1 to P5. Consequently, the Mₗ and pI of the glycoproteins were changed. Note that the five proteins specifically present in human maculae with ARM (P1 to P5, Fig. 1A) have totally disappeared.
Furthermore, we found 88% (7/8) identity in eight N-terminal amino acids of P2 (Table 2) compared with the interphotoreceptor retinoid-binding protein (IRBP) precursor (amino acids 942 to 949) with a p value of 0.93. We could not detect the mature IRBP (M, 145000, pI 4.4 to 4.8) in any of the silver-stained gels. The first 21 N-terminal amino acids of protein spot 6 (P6, Fig. 1A, Table 3) showed a 90% (19/21) correspondence with the N-terminal amino acid sequence of the β-chain of creatine kinase with a p value of 10^-5. This protein was present in all maculae, and its M (43800) was used as an internal molecular weight marker. P1, P4, and P5 could not be sequenced (Table 3).

All five protein spots were N-linked glycoproteins as revealed with N-glycanase digestion of the macular samples (Fig. 2).

Apart from the mentioned five proteins, the protein patterns of extracts of healthy maculae and maculae with ARM were highly reproducible and were almost identical to each other (Fig. 1A, B). Also, the interassay and intra-assay results were highly reproducible for one sample. The protein patterns from maculae with higher post mortem times of 10 to 15 hours (six samples) and maculae with post mortem times up to 10 hours (19 samples) appeared to be similar; thus, we included all samples in our results (Table 1 and 2). These patterns were assessed both visually and by computer-assisted scanning of the gels.

Staining of tissue sections with Ab against haptoglobin (both α-chain and β-chain) revealed specific staining in the lumina and in the innermost layer of the blood vessels in both maculae with ARM and in control maculae. There was no difference in the anti-haptoglobin histopathologic staining pattern between control maculae and maculae with ARM. However, a quantification of haptoglobin content is not possible with this technique. Neither drusen nor BLD stained with Ab against haptoglobin.

Staining with Ab against IRBP showed specific staining of the interphotoreceptor matrix in all samples, but no specific staining was observed in drusen or BLD.

**DISCUSSION**

In this study, the protein composition of human maculae was determined. The total protein amount of one human macula is only about 1 mg, and fresh human donor eyes are hard to obtain, so it is obvious that techniques with a high resolution capacity had to be used. With the silver staining technique, nanograms of proteins can be detected. This implies that we can only detect the main proteins in the tissue with this technique, and any change in minor proteins cannot be detected. About 500 proteins were analyzed by the computer-imaging system.

A significant increase in the expression of five proteins in human maculae with either BLD (Class 2 and 3) or drusen was observed compared with control maculae (Fig. 1A, B, Table 1). Consequently, these proteins are suspect to be related to ARM. However, it could not be determined whether any of these five proteins were significant components of drusen or BLD. They might have originated from histologically less obvious changes that coincide with ARM, e.g., in the choriocapillaris or in the outer retina. Because the staining intensity of P1 to P5 was stronger in maculae with drusen Class 1 and no BLD (Subjects 10 and 11, Table 1) than in maculae with BLD Class 2 or Class 3 (Subjects 8 and 9) and no drusen, it could be speculated that drusen are of more importance than BLD in respect of these proteins (Fig. 2).

Glycoprotein P3 had a 93% identity with the first 15 N-terminal amino acids of the β-chain of haptoglobin (p = 10^-5). The Mr and pI of glycoprotein P3 (Table 3) were both identical with the Mr and pI of the β-chain of haptoglobin (26). Consequently, we characterized this protein as haptoglobin β-chain. Haptoglobin is a carrier for free hemoglobin in the plasma and prevents the heme from becoming oxidative and the hemoglobin from being wasted through the kidneys. Haptoglobin is known to be anti-oxidative and is also one of the acute phase proteins (27). Furthermore, haptoglobin is assumed to be angiogenic (28).

Haptoglobin β-chain is found in fatty streaks and fibro-fatty lesions in the aortic intima in atherosclerosis (29). So it is possible that the increase in haptoglobin β-chain found in this study might be correlated with atherosclerosis. With aging, there is an increase in calcifications (6, 30) and lipids (20, 31, 32) in Bruch’s membrane, the morphologic boundary between the retina and the choriocapillaris. Recently, a relationship between ARM and atherosclerosis has also been found in an epidemiologic study (33). These authors found an increased percentage of atherosclerotic plaques, but no differences in vascular thickness, in the aorta bifurcation and carotid artery in patients with ARM. Furthermore, a relationship between ARM and sclerosis of the choriocapillaris has been found in a clinico-pathologic study (2). In neovascular AMD, new vessels grow from the choriocapillaris into the subretinal space. Haptoglobin has a potential angiogenic role, and we have found haptoglobin in the blood vessels of the choroid, so it might be that haptoglobin β-chain is one of the angiogenic factors in this process.

Glycoprotein P2 had a 88% identity with eight amino acids of the IRBP precursor (Table 3). Normally, IRBP is only found at the apical side of the RPE in the interphotoreceptor matrix and is produced by the photoreceptor cells (34, 35). The amino acid sequence of P2 was not statistically significant when compared with the IRBP precursor, so we could not conclude homology. Nevertheless, it could be feasible that there is an increased or malproduction or a malmetabolism of IRBP in ARM. A possible reason for an increased production could be that much of the IRBP is bound to accumulating derivates of retinol in the RPE (e.g., in lipofuscin granules) and is not available for its normal transport function. As a result of both hypotheses, the photoreceptors will presumably produce more IRBP to preserve a sufficient concentration of IRBP in the interphotoreceptor matrix.
Haptoglobin β-chain and IRBP are both N-linked glycoproteins. With N-glycanase treatment of the macula extracts, we proved that P1 to P5 are N-linked glycoproteins as well (Fig. 2). Glycoproteins P4 and P5 were either N-terminally blocked, or the amounts were too small to investigate by sequence analysis. However, they are likely to be isoforms of haptoglobin β-chain because haptoglobin β-chain normally appears as three to eight closely related protein spots on two-dimensional electrophoresis (26). We were also unable to obtain a sequence of glycoprotein P1 for the same reasons but have no clue to the identity of this protein.

In conclusion, we found a higher amount of five glycoproteins in human macular tissue with ARM than in age-matched control eyes. Little is known about the significance of these proteins in regard to ARM. However, the positive identification of one of these proteins as the haptoglobin β-chain and its relation with angiogenesis and neovascular growth might fit in the concept about the pathophysiology of ARM.

METHODS

CHEMICALS

All equipment, chemicals, and buffers were purchased from Millipore Corporation (Etten-Leur, The Netherlands), unless otherwise described.

TWO-DIMENSIONAL ELECTROPHORESIS

The choroid and retina, frozen together in liquid nitrogen, were defrosted and homogenized on ice in 0.5 ml of 1 mM phenylmethylsulfonylfluoride/Mili-Q water (Sigma Chemical Company, St. Louis, MO), aliquoted in 25 μl, and stored in a −80°C freezer. Homogenized material (50 μg of protein in 25 μl) was precipitated with 25 μl of acetone for 20 minutes at −20°C and was centrifuged at 12,000 rpm for 5 minutes in an Eppendorf microcentrifuge at 4°C. The pellet was solubilized with 25 μl of sample buffer A (0.3% SDS, 200 mM dithiothreitol, 28 mM Tris-HCL, and 22 mM Tris-base) and was heated for 4 minutes at 90°C. Two-dimensional electrophoresis was performed with the Millipore Investigator system. We used ampholytes with a pH range from 3 to 10 for the first dimension. For the second dimension, 10% Duracryl gels were used. Glyceraldehyde-3-phosphate dehydrogenase (GADPDH) carbamylates (Pharmacia, Woerden, the Netherlands) and molecular weight standards (Gibco, Gaithersburg, MD) were used as isoelectric focusing and molecular weight markers, respectively. Albumin was used as an internal reference for protein content. Proteins in two-dimensional gels were visualized with a silver staining technique. The Mᵦ and isoelectric point (pI) values of the proteins were calculated by a Millipore computer-imaging system. All steps were according to the manufacturers’ instructions.

PREPARATIVE TWO-DIMENSIONAL ELECTROPHORESIS

Preparative two-dimensional electrophoresis was performed to obtain enough purified protein for N-terminal amino acid sequence analysis. Therefore, 250 μl of homogenized material (500 μg of protein) was precipitated with cold acetone for 20 minutes and was centrifuged at 12,000 rpm for 5 minutes in an Eppendorf microcentrifuge. The pellet was solubilized with 250 μl of sample buffer A and was heated for 15 minutes at 60°C. First- and second-dimension electrophoresis were performed as described above, according to the manufacturers’ description. Additionally, we added 0.1 mM natriumthioglycolaat to the upper running buffer in the second dimension. After electrophoresis, the gels were blotted on a polyvinylidene difluoride membrane (Immobilon-P), and blots were subsequently stained with filtered (0.45 μm) Coomassie Brilliant Blue R-250 (Sigma). Protein spots of interest were cut out and analyzed.

AMINO ACID SEQUENCE ANALYSIS

N-terminal amino acid sequences of proteins were determined on a protein-sequencing system (Model 473A, Applied Biosystems, Foster City CA). A computer-assisted comparison of the N-terminal amino acid sequences with known sequences in the Swiss Protein Data bank was made. To verify if the found matches were statistically significant, calculations of the p values were made according to Karlin et al. (36).

DETECTION OF N-LINKED GLYCOPEPTIDES

To determine asparagine-linked glycoproteins, samples were treated with N-glycanase (Genzyme Corporation, Boston, MA) before loading, according to Tarentino et al. (37).

IMMUNOHISTOCHEMISTRY

Results pointed to increased levels of haptoglobin and IRBP, so paraffin sections of the maculae were examined for the presence of these proteins. Paraffin sections (5 μm) were mounted on 3-amino-propyltri-oxy-silane (Sigma)-coated glass slides. After deparaffinization and rehydration, they were placed in 3% H₂O₂/methanol for 25 minutes to eliminate endogenous peroxidase activity. The slides were placed in a Sequenza Immunostaining Workstation (Shandon Scientific Ltd, Astmo Runcorn Cheshire, England) and were incubated for 1 hour with rabbit-polyclonal Ab against human IRBP (gift from the Department of Ophthalmic Immunology of the Netherlands Ophthalmic Research Institute, Amsterdam) or with goat-polyclonal Ab against human haptoglobin (Beckman, Fuller-ton, CA). Subsequently, the slides were incubated for 30 minutes with peroxidase-labeled swine-anti-rabbit or rabbit-anti-goat secondary Ab (Dako, Glostrup, Denmark), respectively. The slides were rinsed with PBS (pH 7.6) between all incubation steps. Finally, the slides were stained for 7 minutes with 3,3-diaminobenzidine (Fluka Chemica, Oud-Beijerland, The Netherlands) and counterstained with Mayer’s hematoxylin.

STATISTICAL ANALYSIS

The Student’s t test was used to compare the differences in age between the cases with ARM (n = 9) and controls (n = 7) to rule out any change in protein composition correlated with age.

Differences in amount of a specific protein between maculae with ARM and control maculae were analyzed by subdividing the amount of protein in two groups. Albumin was taken as an internal control for total protein content. No, or just visible, staining was arbitrarily called low concentration. The group with the higher protein concentration was defined as those with distinct staining. Differences between these two groups were tested using Fisher’s exact test.

The correlation of the protein composition between the left and right eye of the individual cases (n = 9) was tested with Spearman’s rank correlation tests.

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