Online supplement to:

Activation of the Alternative Complement Pathway in Vitreous is Controlled by Genetics in Age-related Macular Degeneration

Kelly M. Loyet,¹ Laura E. DeForge,¹ Kenneth J. Katschke, Jr. ¹, Lauri Diehl,¹ Robert Graham,¹ Lily Pao,¹ Lizette Sturgeon¹, Sock-Cheng Lewin-Koh,¹, Joe G. Hollyfield,² and Menno van Lookeren Campagne¹

¹Genentech, Inc., South San Francisco, CA and ²Cleveland Clinic, Cleveland, OH

Eye Dissection Technique Each donor eye was placed in a customized holder to stabilize the globe allowing efficient removal of the anterior segment. The holder consisted of a 4 cm thick 20 × 15 cm rectangular Lucite plate in which a 4-cm diameter spherical depression 2-cm deep was located near the center of the plate. The Lucite base was overlain with a 20 × 15 cm stainless steel plate (0.75-cm thick) containing a 3.5-cm diameter hole in register with the spherical depression in the Lucite plate below. The stainless steel plate was connected at one end of the minor dimension of the Lucite plate with a hinge. With the hinged plate at right angle to the Lucite surface, the intact donor eye was positioned in the spherical depression with the optic nerve down and the cornea up. The hinged plate was lowered so that the cornea was centered in the circular opening. A 28-cm Thomas Tissue Slicer Blade was inserted between the Lucite base and the stainless steel plate and a cut through the eye was made behind the
limbus for removal of the anterior segment. The vitreous was then poured from the posterior eye cup into a 15-mL plastic tube and capped. The Bruch’s membrane–choroid (BM/C) interface was removed, laid flat on a wax substratum, and 4-mm trephine discs were isolated from specific areas of the macula.

Controls for Immunohistochemical Staining

Chinese hamster ovary (CHO) cells were opsonized with hamster immunoglobulin G and incubated in a mixture of 50% human serum and 40% rat type II collagen. The serum was either normal human serum, factor D (FD)- or factor B (FB)-depleted serum, or serum was omitted, as indicated. Sections cut through the cell pellet were immunostained with antibodies to the various complement proteins.

Protein Extraction From BM/C and Western Blot Analysis

For protein extraction of Bruch’s membrane–choroid (BM/C) trephine discs, 4 mm in diameter and 300 ± 55-µm thick (mean ± SD; based on histology sections), were dissected and incubated for 10 minutes on ice in 1× cell lysis buffer (Cell Signaling Technologies, Danvers, MA), 1× protease inhibitor cocktail (Set 1, Calbiochem, EMD Biosciences, La Jolla, CA), and 4 nM phenylmethylsulfonyl fluoride (Roche, Indianapolis, IN). There was no difference in trephine disc thickness comparing category 1, 3 and 4 eyes. The mixture was homogenized on ice for 1 minute, sonicated three times for 30 seconds, and centrifuged for 10 minutes at 14,000 rpm at 4°C. The supernatant was collected and stored at −80°C. To assure all complement proteins were recovered in the soluble fraction, the remaining insoluble fraction was boiled in 1%
sodium dodecyl sulfate (SDS) sample buffer and remaining proteins in the boiled fraction detected by Western blot analysis (Figure S2). Briefly, lysates were reduced in Laemmlı’s buffer and β2-mercaptoethanol (BioRad, Hercules, CA), boiled for 5 minutes, loaded (17 µl) on 4-20% gradient Tris-glycine precast gel (Invitrogen, Carlsbad, CA), transferred to 0.2 µm nitrocellulose membrane (BioRad, Hercules, CA), and blocked in PBS-0.1% Tween-20/5% milk for 1 hour at room temperature. Western blot analysis was performed using horseradish peroxidase–conjugated antibodies to C3 (1:5000, goat-anti-human C3 [MP Biomedicals, Aurora, OH]), FD (0.5 µg/ml, [Genentech, Inc.]) and FB (0.5 µg/ml, [Genentech, Inc.])

**Preparation of Plasma from Normal Human Donors**

Human plasma was prepared from whole blood obtained with informed consent from the donors. Since complement activation can readily occur ex vivo, blood was collected in EDTA Vacutainers (10 mM final EDTA; BD, Franklin Lakes, NJ), the tube was turned 4 – 5 times to ensure EDTA mixing, and 50 mg/mL Futhan (BD Pharmingen), a broad-spectrum inhibitor of complement protease activity, was added. Alternatively, EDTA/Futhan tubes (Venoject) were used. The blood sample was placed on ice or at 4º C immediately, then centrifuged for 15 min at 1500 x g, followed by removal of plasma.
**Figure S1.** Complement proteins are efficiently extracted from BM/C preparations. Western blot analysis of complement proteins in supernatants from lysates of BM/C and in insoluble BM/C pellets boiled in sodium dodecyl sulfate sample buffer. Purified complement proteins are shown in the right panels for reference. FB = factor B; FD = factor D.
**Figure S2.** Specificity of the immunohistochemical staining procedure using (A) Isotype control and specific antibody staining for C3, C4 and FB on serial sections through BM/C of a category 1 eye, (B) staining with isotype control and anti-FD on two consecutive sections through the BM/C of a category 3 eye. Arrowheads indicate FD-positive small drusen (C) staining with isotype control and anti-FB on two consecutive sections through the liver, (D) Chinese hamster ovary cell pellets were treated with normal human serum (+ C), complement-depleted serum (– C), or without serum (0 C) and stained following a protocol similar to that for sections through BM/C described above.
Figure S3. Comparison of factor D (FD), factor B (FB), and C3 protein concentration in plasma from control subjects, and in vitreous and Bruch’s membrane–choroid (BM/C) lysates from category 1 donors. (A) Graphs representing mean ± SEM of 24 (plasma and BM/C) and 30 (vitreous) samples. (B) Table indicates concentration of complement proteins and molar ratio’s of FB and C3 compared with FD (set as 1).

A

![Graphs representing mean ± SEM of 24 (plasma and BM/C) and 30 (vitreous) samples.](image)

B

<table>
<thead>
<tr>
<th>Source</th>
<th>Protein Concentration</th>
<th>Molar Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FD</td>
<td>FB</td>
</tr>
<tr>
<td>Plasma (nM)</td>
<td>42 ± 11</td>
<td>1647 ± 440</td>
</tr>
<tr>
<td>Vitreous (nM)</td>
<td>17 ± 15</td>
<td>30 ± 29</td>
</tr>
<tr>
<td>BM/C (fmol/mm²)</td>
<td>1.3 ± 1.4</td>
<td>0.9 ± 1.0</td>
</tr>
</tbody>
</table>