Metabolic Impacts of Cigarette Smoke on RPE and Muller Cells of Complement Compromised Mice

Alexandra D. Butler

Alexandra D. Butler and Bryan W. Jones

Ophthalmology

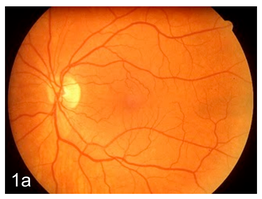
Bryan W. Jones

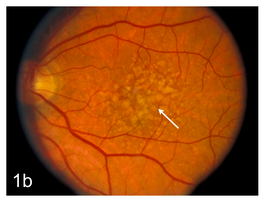
THE UNIVERSITY OF UTAH

Purpose

Age-related macular degeneration (AMD) is one of the largest causes of blindness worldwide. Cigarette smoking is known to be the single largest risk factor for AMD, aside from age. Several of the main genetic risk factors for AMD are polymorphisms occurring in complement genes involved in the alternative, classical and common terminal pathways.

To better understand the metabolic impact of smoking on the retina, computational molecular phenotyping (CMP) was used to examine the effects of cigarette smoke on wild type (WT) mouse retinas and retinas of mice in which either the alternative pathway (complement factor B, CfB) or the common terminal pathway (complement component 3, C3) was removed. Specifically, Müller Cells, Retinal Pigment Epithelium (RPE), and photoreceptor Inner and Outer Segments were examined across the aforementioned conditions.



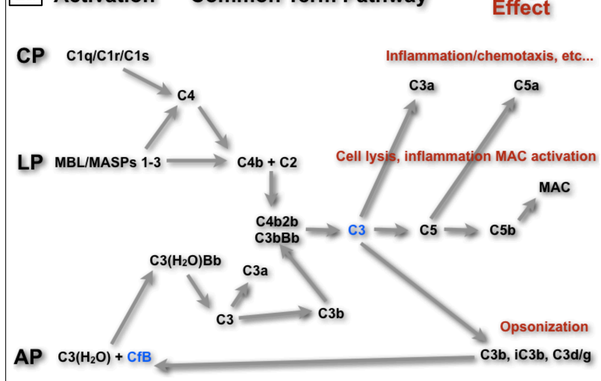


Background

AMD

Shown above are images of a normal human retinal fundoscopic exam illustrating normal color, pigment and vascular distribution (1a), and a human fundoscopic image showing classic extensive drusen commonly observed in AMD (1b). Drusen contain numerous proteins associated with the compliment cascade, related to the process of inflammation or its aftermath. Research has shown that the complement cascade is involved in AMD.

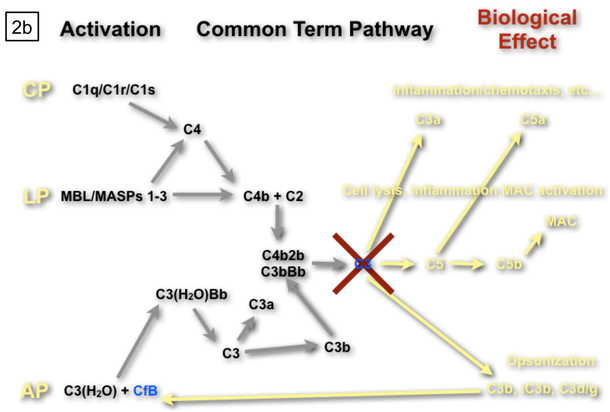
Recent research by DeAngeles, Curcio, Hageman and others has shown that AMD may not necessarily be a disease of the retina, but that retinal tissue manifests the disease in response to systemic insults. We are interested in how these effect may be visible in retinal metabolic measures and in particular, the AMD retina. As there are no accepted models of AMD with drusen, we focused on the biggest risk factor for AMD, smoking.

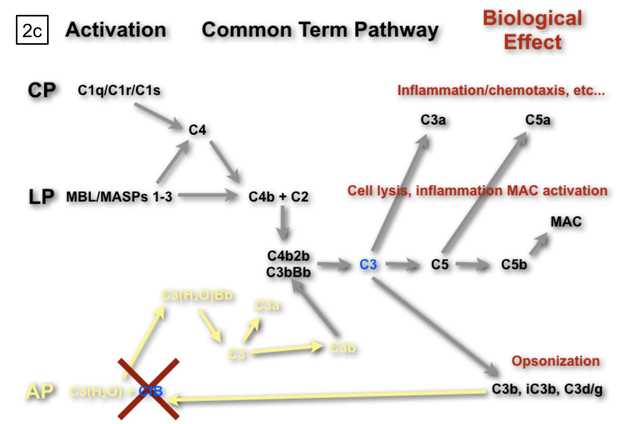


Complement Cascade:

The complement system assists the immune system in clearing pathogenic antigens from the body. Complement can be recruited through three separate pathways: the classical, lectin, and alternative pathways (2a). Activation of the classical and lectin pathways results in inflammation, chemotaxis, cell lysis, membrane attack complex (MAC) activation, and other aspects associated with immune responses. The alternative pathway enhances the phagocytosis process through a feedback or amplification loop of the opsonization process or enhancing the phagocytosis of antigens.

Removal of C3 eliminates all biological effects of the complement system (2b). Removal of CfB eliminates the amplification loop that generates the full-blown complement attack (and the spontaneous turnover and initiation by the alternative pathway, but it isn't yet known what role this plays in the eye) (2c). We have not yet found anything that is purely dependent upon the alternative pathway.

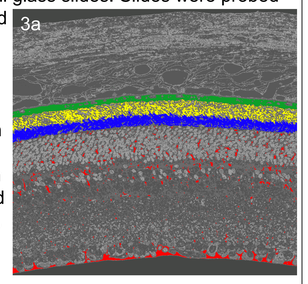




Methods

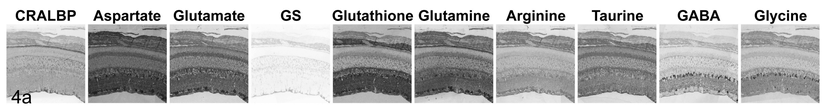
While smoking is common, obtaining post-mortem tissues from human subjects in a timely fashion is problematic. Therefore, in collaboration with Dr. Baerbel Rohrer's laboratory at the Medical University of South Carolina (MUSC), we examined the effect of cigarette smoke on the mouse eye. The mouse eye is remarkably similar to the human eye, with very similar small molecular values in retinal tissues, and serves as a reasonable model for this evaluation. Another advantage of a mouse model is that it allows for alterations in the gene sequence, letting us look at the alterations in the complement cascade and compare WT, CfB knockout, and C3 knockout mice.

Mice were exposed to cigarette smoke generated using an automated cigarette-smoking machine (Model TE-10, Teague Enterprises, Davis, CA) burning 3R4F reference cigarettes (2.45 mg nicotine per cigarette). Mice were exposed for 6 hours/day, 5 days/week for 6 months. Age matched room filtered air exposed mice were used as controls. Eyes were enucleated immediately post-mortem, fixed in 1% paraformaldehyde, 2.5% glutaraldehyde, dehydrated in graded methanol, embedded in eponates as stacks of 6 and thin sectioned at 180 nm into 10 well glass slides. Slides were probed with antibodies generated against small molecules of interest and histologically analyzed with CMP. Signals were detected and intensified with silver, then digitally captured. The captured images were registered and clustered to mask out specific cell classes for quantitative statistical analysis. Unsupervised clustering methods, like k-means clustering, allow us to deal with complex data and segment it into manageable classes that can provide statistical measures of separability. We can then focus in on different mathematical cell classes. Here we have considered Photoreceptor Inner Segments (blue), Outer Segments (yellow), RPE (green), and Müller Cells (red) (3a).



Results

Shown below are some images taken from a wild type mouse that has been exposed to cigarette smoke (4a). The sections are cut thin enough so that each one of the labels shows signals of molecules from the same cells. This allows for determination of signals of each cell type across experimental conditions.



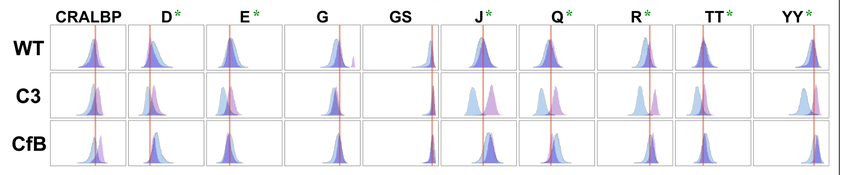
Alterations in retinal small molecule signatures were observed in smoked retinas compared to non-smoked retinas from mice in WT, CfB knockout, and C3 knockout conditions. Histograms were then created from the grey-scale profile for the cell class of interest, which can be used to analyze signal densities of the molecules of interest. These are log plots on the horizontal axis, so small differences are actually substantial. Shown are the concentration profiles for the Inner Segments, Outer Segments, Müller Cells, and RPE. Plots of particular interest or significance are indicated by a green asterisk (*).

Inner and Outer Segments of WT smoked retinas both demonstrated changes in small molecule levels relative to non-smoked retinas. In the Inner Segments, the complete complement, C3 knockout, mice had the more dramatic effects, whereas the CfB mice showed more restrained changes. This pattern was especially evident in D, E, J, Q, R, TT, and YY. This is a predictable response, as the increased inhibition of the complement cascade would be expected to create more dramatic alterations to cell stress response pathways.

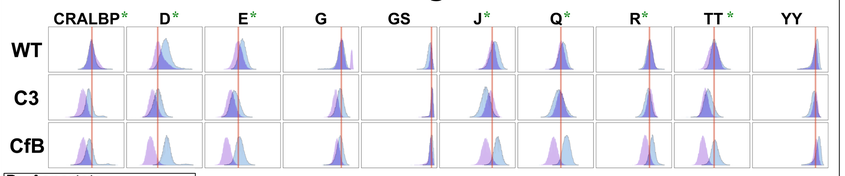
In the Outer Segments, signal changes between non-smoked and smoked conditions in D, E, J, Q, R, and TT progressively increased in the retinas of WT, C3, and CfB knockout mice indicating increased response profiles to cell stress. The fact that there are significant differences in the outer segments is surprising given how little room there is in a presumptive normal outer segment for anything other than rhodopsin. CfB knockout mice seem to have the most dramatic effect on cell stress response pathways in the outer segments. This is somewhat paradoxical, as the more complete inhibition of the complement cascade in the C3 knockout would be expected to have a more dramatic effect that the CfB knockout of the feedback loop.

Non-Smoked Smoked

Inner Segments



Outer Segments

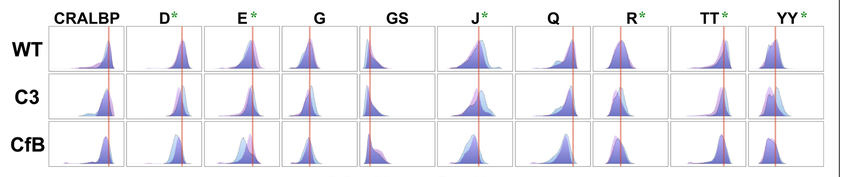


D - Aspartate
E - Glutamate
G - Glycine
GS - Glutamine Synthase
J - Glutathione
Q - Glutamine
R - Argenine
TT - Taurine
YY - GABA

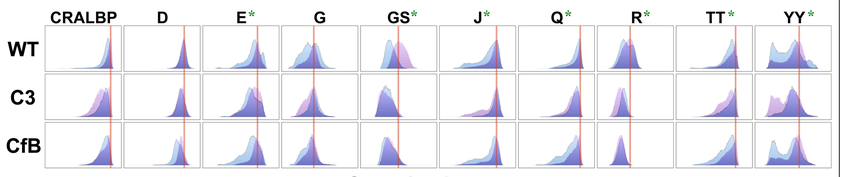
Both Müller Cells and RPE of WT smoked retinas demonstrated changes relative to non-smoked retinas. In RPE, non-smoked C3 and CfB knockout samples showed decreases in D, E, J, R, TT, and YY compared to non-smoked WT. Smoking resulted in increases across these small molecules for C3, while CfB showed no change, or further decreases when compared to non-smoked samples. In the Müller Cells, similar to RPE, C3 and CfB knockouts showed decreases in GS, R, TT, and YY relative to WT samples. Smoked C3 had some increase in these levels, nearing original non-smoked WT levels, and smoked CfB showed little-to-no changes, or further decrease.

Separate from the shifts in small molecule levels, the variance of metabolic signals within Müller Cell class increased. Under pathological conditions, like exposure to cigarette smoke or modifications to the functionality of the immune system, cell populations normally described by narrow small molecule signal profiles become more disorganized. Signal variance of CRALBP, E, G, J, Q, and TT increased in both non-smoked knockouts relative to WT samples. However, under smoked conditions, the variance of these small molecules in the C3 samples decreased, while that of CfB knockouts continued to increase. This indicates a disruption, to some degree, of the mechanisms regulating those small molecule levels and the cells stress response pathway, particularly under the smoked CfB knockout condition.

RPE



Müller Cells



Conclusions

In response to cigarette smoke exposure; we see substantial metabolic changes in photoreceptors. Glutamate, glutamine, glutathione and taurine are known to be involved in cell stress response & osmoregulation. Glutamine and glutathione increased in photoreceptor cells upon smoke exposure. Eliminating essential components of the complement system, a cascade required for the maintenance of the immune privilege of the eye, appears to exacerbate responses to cigarette smoke in oxidative damage response related pathways. The complete blockade of the complement system via the common terminal pathway (C3 knockout) has a more substantial impact on the inner segments or photoreceptor cell bodies than a partial blockade of the complement system via the alternative pathway (CfB knockout).

The importance of the RPE cannot be understated in the maintenance of photoreceptors while Müller Cells are perhaps best positioned to respond to environmental insults. Glutathione and glutamine are up-regulated in cells undergoing cellular/oxidative stress while arginine up-regulation may reveal alterations in gene expression as cells alter metabolism to stabilize gene translation. The changes we see in the Müller Cells and RPE of the levels of these small molecules, and of signal variance, seem to indicate that there is some response to the oxidative stress of cigarette smoke that is functional in the C3 knockout that is disabled in the CfB knockout.

Furthering our understanding of complement-dependent metabolic alterations in the eye might aid in our understanding of AMD pathology and may open new avenues for novel treatment strategies.