Molecular Vision 2015; 21:264-272 <http://www.molvis.org/molvis/v21/264>
Received 2 September 2014 | Accepted 11 March 2015 | Published 13 March 2015

CFH Y402H polymorphism is associated with elevated vitreal GM-CSF and choroidal macrophages in the postmortem human eye

Jay Ching Chieh Wang, Sijia Cao, Aikun Wang, Eleanor To, Geoffrey Law, Jiangyuan Gao, Dean Zhang, Jing Z. Cui, Joanne A. Matsubara

(The first two authors contributed equally to this work.)

Department of Ophthalmology and Visual Sciences, University of British Columbia, Vancouver, BC, Canada

Purpose: Age-related macular degeneration (AMD) is the leading cause of irreversible blindness in people 50 years of age or older in developed countries. The homozygous CC genotype in the complement factor H *(CFH)* Y402H single nucleotide polymorphism (SNP; rs1061170) is widely recognized as a risk factor for the development of AMD. In this study, we examined vitreal levels of granulocyte macrophage colony-stimulating factor (GM-CSF), a hematopoietic cytokine, and macrophages in the choroid of postmortem human eyes genotyped for the *CFH* Y402H SNP.
Methods: Twenty-two pairs of postmortem, non-diseased, human donor eyes were obtained. The vitreous and retinal tissues of the left eyes were collected for GM-CSF level measurement and *CFH* Y402H genotyping, respectively. The right eyes were paraffin-embedded and sectioned for immunohistochemistry using a macrophage and microglia marker, CD68. Cell cultures of RPE cells were stimulated with complement C3a, C5a, 4-hydroxynonenal (HNE), or tumor necrosis factor alpha (TNF-α), and GM-CSF expression was measured with a suspension assay or quantitative PCR.
Results: Eyes genotyped with the CC or the CT risk variant of the *CFH* Y402H SNP showed significantly increased levels of GM-CSF in the vitreous compared to eyes with the protective TT variant (mean ± standard error of mean, 607.54±85.83 pg/ml or 656.32±15.20 pg/ml versus 286.69±81.96 pg/ml, $p<0.05$). The choroid of eye tissues genotyped with the CC variant showed higher levels of CD68 immunoreactivity than the tissues genotyped with the TT variant ($p<0.05$). The GM-CSF levels detected in the supernatant of RPE cells in culture treated with HNE or TNF-α were significantly higher compared to the non-treated control (145.88±5.06 pg/ml and 149.32±3.76 pg/ml versus 123.27±4.05 pg/ml, $p<0.05$). Furthermore, the gene expression of GM-CSF detected in the lysate of RPE cells stimulated with complement C3a or C5a showed significantly increased fold changes compared to the non-treated control (C3a: 2.38±0.31 fold, $p<0.05$; C5a: 2.84±0.54 fold, $p<0.01$).
Conclusions: Our data showed a relationship between the *CFH* Y402H polymorphism and GM-CSF levels in the vitreous and accumulation of choroidal macrophages in the postmortem eye. These data suggest that the at-risk variant of the *CFH gene* may contribute to the dysregulation of proinflammatory cytokines locally in the eye.

Age-related macular degeneration (AMD) is the leading cause of irreversible central blindness among people aged 50 and above in developed countries [1-4]. AMD is a progressively degenerative disease of the retina characterized by dysfunction of RPE cells and associated photoreceptor loss [2,5]. Although the mechanisms leading to the development of AMD are not well understood, it is believed that the cause is multifactorial and that genetics, chronic local inflammation, and oxidative stress have been implicated in the pathogenesis of AMD [6-8].

Earlier genome-wide association studies reported that the single nucleotide polymorphism (SNP) Y402H (rs1061170, sequence: T1277C) in the gene coding complement factor H (CFH; OMIM 610698) is associated with AMD [9-11]. The homozygous CC variant of the *CFH* Y402H SNP is associated with an approximate sixfold increased risk of developing AMD, compared to the TT variant [9-12]. The mechanism by which the at-risk CC variant confers an increased risk for AMD is still under investigation. Previous studies, including our own, have suggested that the *CFH* Y402H at-risk variant is associated with elevated levels of complement activation products and classic proinflammatory cytokines, including tumor necrosis factor alpha (TNF-α) [13-16]. Moreover, a higher level of oxidative stress markers appears to be associated with polymorphisms in *CFH* [17].

In this study, we focus on granulocyte macrophage colony-stimulating factor (GM-CSF), a growth factor that promotes the survival and activation of microglia and

Correspondence to: Joanne A. Matsubara, Department of Ophthalmology and Visual Sciences, Eye Care Centre, 2550 Willow Street, Vancouver BC V5Z 3N9, Canada; Phone: 604 875-4383; FAX: 604 875-4663; email: jms@mail.ubc.ca

macrophages and its relationship with the *CFH* Y402H polymorphism in the postmortem human eye and RPE cell cultures [18]. Previous histological studies showed microglia and macrophages are associated with AMD lesions, although the role of these cells is not clear [19]. In animal studies, transgenic mice that lack *CFH*, or express the human risk variant of *CFH*, demonstrate increased levels of macrophages and microglia in the kidney, brain, and central retina compared to the wild-type controls [20-22]. In addition, macrophages and microglia differentiate and proliferate when stimulated with GM-CSF [18,23,24]. Given this evidence in transgenic mice, we were interested in understanding the relationship between the *CFH* Y402H genotype and GM-CSF levels in the postmortem human eye. We hypothesize that eye tissues from donors genotyped with the at-risk CC variant would exhibit higher levels of GM-CSF and accordingly, more macrophages or microglial cells compared to the eye tissues from donors with the protective TT variant. Given the evidence that *CFH* polymorphisms interact with oxidative stress and proinflammatory molecules, we also investigated the role of 4-hydroxynonenal (HNE), TNF-α, and complement activation products C5a and C3a in GM-CSF regulation in the RPE in vitro.

METHODS

Postmortem donor eye tissues: Twenty-two pairs of postmortem donor eyes were obtained from the Eye Bank of British Columbia (Vancouver, Canada). The study protocol was approved by the Clinical Ethics Research Board of the University of British Columbia and strictly adhered to the Declaration of Helsinki. All eye specimens used in this study were normal. Eyes from donors with the following pathologies were excluded for use in this study: evidence of local or systemic infection, progressive central nervous system pathologies, systemic diseases of unknown origin, lymphoproliferative disorder, myeloproliferative disorders, or any intrinsic eye disease. The right eyes were paraffin-embedded to obtain 6 µm sections for immunohistochemistry. The left eyes were dissected to collect the vitreous and the retina. Briefly, each globe was incised circumferentially at the pars plana to remove the anterior segment. Approximately 3.5–4 ml of vitreous was aspirated by inserting a 20 gauge needle attached to a 5 ml syringe into the vitreous chamber. The vitreous samples were centrifuged at 1500 ×g for 15 min at 4 °C, and the supernatant was collected, aliquoted, and stored at −80 °C until use [25-28]. The neuroretina tissues were carefully removed from the RPE and choroid and cut into 5 mm × 5 mm blocks for DNA extraction and *CFH* genotype analyses. The length of time between death and the collection of postmortem human eye tissues varied due to individual institutional or hospital procedures. To investigate whether the variability in postmortem collection time affected the GM-CSF level, we conducted a Pearson correlation test and found that there was no relationship between the GM-CSF level and the collection time (r=0.2769, p=0.237, Appendix 1).

Total genomic DNA isolation and CFH Y402H genotyping: The methods for isolating total genomic DNA and genotyping followed those used in our earlier study [28]. Total DNA was isolated from the retinal tissues of each donor following the phenol-chloroform-isoamyl method, and then amplified with PCR for the selected fragment of the *CFH gene* spanning the Y402H SNP locus (rs1061170). The forward and reverse primers used were 5′-AGTA ACT TTA GTT CGT CTT CAG-3′ and 5′-ATC TTC TTG GTG TGA GAT AAC G-3′, respectively. The amplified PCR products were then purified and sequenced (Genewiz, South Plainfield, NJ). The sequencing primer for *CFH* was 5′-ACT TTA GTT CGT CTT CAG-3′.

GM-CSF level measurement: The GM-CSF levels in vitreous fluids or supernatant from in vitro experiments were measured with a suspension assay (Bio-Rad Laboratories, Hercules, CA). Briefly, 50 µl of standards and diluted samples (1:4) were incubated with the premixed anti-GM-CSF conjugated beads in 96-well filter plates at room temperature, with agitation (1,100 rpm for 30 s and then 300 rpm for 2 h). After washing, the plates were then incubated with 25 µl of diluted biotinylated detection antibody for 30 min at room temperature with agitation, followed by three washes and incubation with 25 µl of streptavidin–phycoerythrin for 10 min at room temperature with agitation. The bead-bound standards and samples were resuspended in 125 µl of Bio-Plex assay buffer and vortexed for 30 s at 1,100 rpm before being analyzed using the Bio-Plex 200 Suspension Array System. The subsequent raw median fluorescent intensity data were captured and analyzed using Bio-Plex Manager software 4.1 (Bio-Rad Laboratories).

The gene expression level of GM-CSF in cells was assessed with quantitative PCR (qPCR). Total RNA was isolated from tissue using the ultRNA column purification kit (Applied Biologic Materials, Richmond, Canada) and reverse transcribed into cDNA using the High Capacity RNA-to-cDNA Master Mix (Life Technologies, Burlington, Canada). RNA quantity and quality were assessed using the NanoDrop 2000c spectrophotometer (Fisher Thermo Scientific, Wilmington, DE). The same quantity of total RNA from each group was used for the reaction. The following GM-CSF primers were used: forward: 5′-AAA GGC TAA AGT TCT

CTG GA-3′; reverse: 5′-CCT GGA GGT CAA ACA TTT C-3′. qPCR was performed on the 7500 Fast SDS (Applied Biosystems, Carlsbad, CA) with the following cycling conditions: 95 °C for 30 s, 50 °C for 30 s, 72 °C for 45 s, 45 cycles. Melting curve analysis was automatically performed immediately after amplification. Each stimulation group was compared to the control group, and the results were expressed in mRNA fold change normalized to the housekeeping gene glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*; OMIM 138400) using the $2^{-\Delta\Delta Ct}$ method. The ΔCt values were subjected to statistical analysis.

RPE stimulation: Human RPE cells were isolated from fetal donor eye tissues for primary culture as previously described [29,30]. Primary RPE cells and ARPE-19 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS; Fisher Scientific, Ottawa, Canada), penicillin (100 U/ml, Fisher Scientific), and streptomycin (100 µg/ml, Fisher Scientific). The primary RPE cells at passage 4 were seeded in a 24-well plate and cultured in complete medium for 20 h. Stimulation experiments were conducted on RPE cell cultures at 90% confluence. The cells were washed twice with PBS (1X; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4). Then, 200 µl of 1× phenol-free minimum essential media (MEM)/F12 medium (Life Technologies) containing either HNE (Millipore, Etobicoke, Canada) at 10 µM or recombinant human TNF-α (R&D Systems, Minneapolis, MN) at 20 ng/ml was added into each corresponding well and incubated for 6 h. C3a or C5a (R&D Systems) at 5 µg/ml was used to incubate the cell culture for 24 h. After incubation, the resulting supernatants or the cell lysate were collected, centrifuged, and stored at −80 °C for the suspension assay or qPCR.

Immunohistochemistry: The immunohistochemical procedures used in this study followed those previously described [30,31]. Briefly, formalin-fixed paraffin-embedded sections were deparaffinized and rehydrated with standard procedures. After heat-induced antigen retrieval in citrate buffer (pH 6.0) for 20 min, sections were blocked with 0.3% H_2O_2 for 15 min and 3% normal horse serum for 40 min. Antibody against human CD68 (1:50, clone PG-M1, Dako, Burlington, Canada) was applied as the primary antibody at 4 °C overnight. Primary antibody omission and non-immune isotype antibodies were used as negative controls. The sections were then incubated in the appropriate secondary antibody and developed in the ABC–AEC system (Vector Labs, Burlingame, CA). The nuclei were counterstained with Mayer's hematoxylin (Sigma Aldrich). The CD68-positive cells were assessed in the choroid and the neuroretina using the 40X objective lens (Eclipse 80i; Nikon, Tokyo, Japan). Only the CD68-positive cells in the choroidal stroma were scored while those in vessel lumens were ignored. The immunoreactive cell numbers in the choroid were counted and then normalized to an area of 0.1 mm^2. The number of CD68-immunoreactive cells was compared between the at-risk CC genotype eyes and the normal TT genotyped eyes.

Statistical analysis: The results were described as frequency (percent) for categorical variables and mean (standard error of the mean [SEM]) for continuous variables. One-way ANOVA (ANOVA) and the post hoc Bonferroni multiple comparison test were used to determine whether the vitreous GM-CSF level, CD68-immunoreactive cell numbers, donor age, and collection time differed among those with the at-risk CC variant, the heterozygous CT variant, and the protective TT variant. The chi-square test was conducted to see if there is a relationship between the genotypes and the sex of the donors. The Student *t* test was used to assess whether the GM-CSF level derived from primary RPE culture changed after exposure to HNE, TNF-α, and C3a and C5a stimulation. The association between the vitreous GM-CSF level and the collection time was assessed using the Pearson correlation. All analyses were conducted with GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). Statistical significance in this study was set at $p<0.05$.

RESULTS

GM-CSF is elevated in the vitreous of eyes with the at-risk homozygous CC variant in CFH: In Table 1, the characteristics of the postmortem donor eyes used in this study are outlined. Briefly, all eye specimens were free of ocular diseases. The mean age of the sample population was 58.5±3.3 years old, and the mean length of time between death and donor eye harvest was 14.3±1.1 h. Ten donors were female, and 12 were male. When categorized by *CFH* Y402H SNP, four donors had the at-risk CC variant, ten had the CT variant, and eight had the protective TT variant.

To determine whether the GM-CSF level differs among the *CFH* Y402H variants, we compared the GM-CSF levels in the vitreous among eyes with the at-risk homozygous CC variant, those with the heterozygous CT variant, and those with the TT variant. The GM-CSF levels differed significantly among donors with CC, CT, or TT variants of the *CFH* Y402H polymorphism (one-way ANOVA, $p<0.01$, Table 2). The GM-CSF level was significantly higher in the vitreous obtained from the donors with CC (n=4, 607.54±85.83 pg/ml) or CT (n=10, 656.32±15.20 pg/ml) variants compared to that obtained from donors with the TT variant (n=8, 286.69±81.96 pg/ml, post hoc Bonferroni multiple comparison test, $p<0.05$,

Table 1. Characteristics of post-mortem human donor eyes.

Sample characteristics	Mean (SEM) or N (%)	
Age (years)	58.5	(3.3)
Collection time (h)	14.3	(1.1)
Gender		
Male	12	(54.5)
Female	10	(45.5)
CFH Y402H genotype		
CC	4	(18.2%)
CT	10	(45.5%)
TT	8	(36.3%)

Figure 1). The vitreous level of GM-CSF was 2.12 and 2.29 times higher in donors with CC or CT variants than those with the TT variants, respectively. This finding is independent of age of donor, sex of donor, and collection time (Table 2).

Macrophages are increased in eyes with the at-risk homozygous CC variant in CFH: GM-CSF is a growth factor that activates monocyte-derived cells such as macrophages and microglia [18,32]. Since higher levels of GM-CSF were found in the vitreous of eyes with the CC variant, we hypothesized that there would be more of these immune cells in the eye of the at-risk CC variant compared to the eyes with the protective TT variant. To examine the level of microglia and macrophages, postmortem human eye sections were immunoreacted with CD68, a known marker for the monocyte lineage [33,34]. Our data revealed significantly more CD68 immunoreactive cells in the choroid of the CC eyes (3.44±0.77 per 0.1 mm^2, n=4) compared to the TT eyes (1.12±0.20 per 0.1 mm^2, n=6, one-way ANOVA and post hoc Bonferroni multiple comparison test, $p<0.05$, Figure 2).

Stimulation of RPE with C3a and C5a promotes upregulation of GM-CSF in vitro: The RPE in the outer retina produces and secretes GM-CSF [35]. In addition, previous literature suggested that the level of complement activation is elevated not only systemically but also locally in the outer retina of donors genotyped with the at-risk CC variant in *CFH* [14,36].

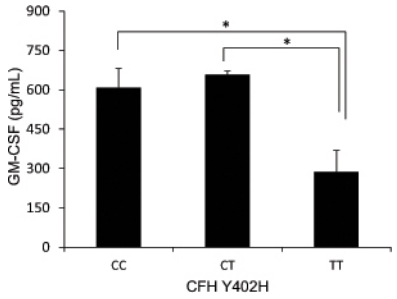

Figure 1. Increased GM-CSF in vitreous was associated with the at-risk variants of the *CFH* Y402H polymorphism. The GM-CSF level differed significantly among donors with CC, CT, or TT variants of the *CFH* Y402H polymorphism (one-way ANOVA, $p<0.01$). The GM-CSF level was significantly higher in the vitreous from donors with CC and CT variants compared to that of donors with the protective TT variant (mean ± SEM, 607.54±85.83 pg/ml, n=4, 656.32±15.20 pg/ml, n=10 versus 286.69±81.96 pg/ml, n=8, respectively, post hoc Bonferroni multiple comparison test, *$p<0.05$).

Therefore, we investigated GM-CSF expression with human ARPE-19 cells stimulated with complement activation products, C3a and C5a. GM-CSF expression was upregulated 2.38 and 2.84 fold by C3a and C5a at 5 µg/ml after 24 h incubation, respectively (C3a: n=3, 2.38±0.31 fold, Student *t* test $p<0.05$; C5a: n=3, 2.84±0.54 fold, Student *t* test, $p<0.01$, Figure 3).

Stimulation of RPE with HNE and TNF-α promotes secretion of GM-CSF in vitro: Apart from complement activation products, C3a and C5a, higher levels of oxidative stress and proinflammatory cytokines were also associated with the *CFH* polymorphism [16,17]. Therefore, we also investigated whether HNE, an agent that promotes oxidative stress, and TNF-α, a proinflammatory cytokine, might affect the secretion of GM-CSF by RPE. Ten micromoles of HNE and 20 ng/ml TNF-α were used to stimulate the primary RPE in vitro, and the level of GM-CSF secreted by the RPE into the culture supernatant after 6-h stimulation was measured. The mean GM-CSF levels secreted by the RPE in the HNE treatment, the TNF-α treatment, and the control group were 145.88±5.06 pg/ml, 149.32±3.76 pg/ml, and 123.27±4.05 pg/ml, respectively (n=3 per group). The GM-CSF levels secreted by HNE- or TNF-α-stimulated RPE were higher compared

Table 2. Descriptive statistics and ANOVA results for vitreous GM-CSF, age and collection time stratified by different genotypes of the CFH Y402H polymorphism.

Variables	CFH Y402H polymorphism						P value
	CC		CT		TT		
	Mean	SEM	Mean	SEM	Mean	SEM	
GM-CSF (pg/ml)	607.54	85.83	656.32	15.20	286.69	81.96	0.0003
Age (years)	60.25	6.84	58.90	4.48	62.50	4.22	0.8505
Collection time (h)	14.50	1.76	16.56	1.58	11.50	2.04	0.1487

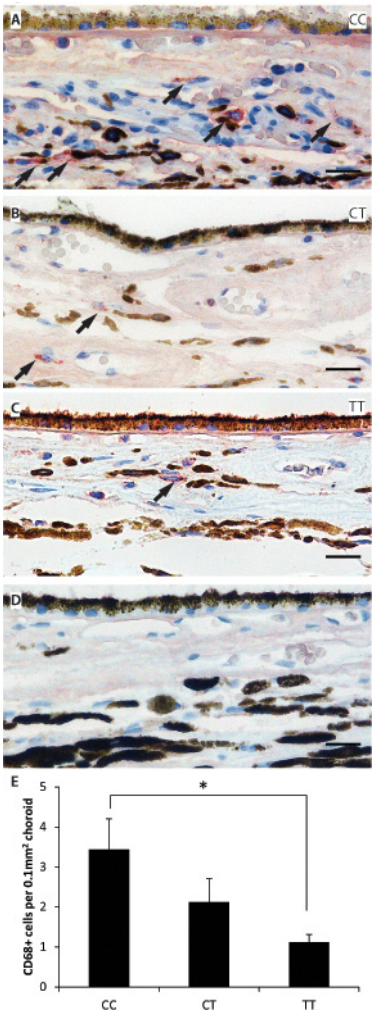

Figure 2. The increased number of macrophages in the choroid was associated with the at-risk variants of the *CFH* Y402H polymorphism. The macrophages in the postmortem human eye sections were immunoreacted with CD68, a known marker for macrophages. The immunoreactivity for CD68 was developed with AEC (red) and counterstained with Mayer's hematoxylin (blue; scale bar: 20 µm). **A**: A representative picture of CD68 immunoreactivity in the outer retina from a donor eye with the at-risk CC variant. **B**: A representative picture of CD68 immunoreactivity from a donor eye with the heterozygous CT variant. **C**: A representative picture of CD68 immunoreactivity from a donor eye with the protective TT variant. **D**: Negative control by primary antibody omission. **E**: The number of CD68-positive cells per 0.1 mm^2 choroid area differed significantly among donors with CC, CT, or TT variants of the *CFH* Y402H polymorphism (mean ± SEM, one-way ANOVA, $p<0.05$). There are more CD68-immunoreactive cells in the choroid of the CC eyes (3.44±0.77 per 0.1 mm^2, n=4) compared to the TT eyes (1.12±0.20 per 0.1 mm^2, n=6; post hoc Bonferroni multiple comparison test, *$p<0.05$).

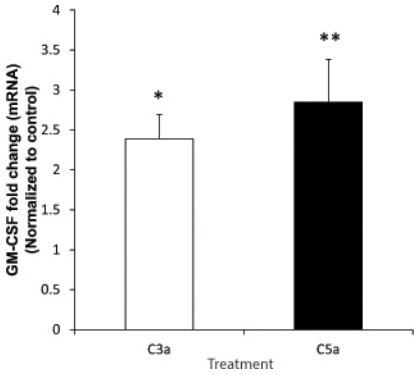

Figure 3. Stimulation with complement activation products upregulated the expression of GM-CSF in the RPE. The expression of GM-CSF in ARPE-19 cells was upregulated 2.84- and 2.38-fold by C5a and C3a at 5 µg/ml after 24 h incubation, respectively (C5a: mean ± SEM, 2.84±0.54 fold, n=3, Student *t* test, **$p<0.01$; C3a: 2.38±0.31 fold, n=3, Student *t* test, *$p<0.05$).

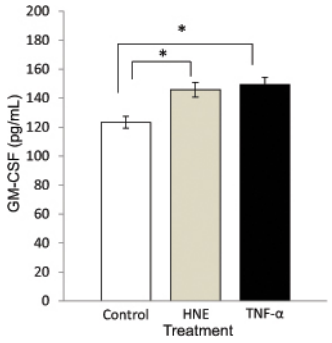

Figure 4. The RPE secreted higher levels of GM-CSF after stimulation with HNE and TNF-α. The GM-CSF secreted into the culture supernatant was increased when primary RPE cells were exposed to 4-hydroxynonenal (HNE), an agent that promotes oxidative stress, at 10 µM for 6 h (mean ± SEM, 145.88±5.06 pg/ml versus 123.27±4.05 pg/ml, n=3, Student *t* test, *p<0.05). In addition, RPE cells stimulated with 20 ng/ml tumor necrosis factor alpha (TNF-α) for 6 h also resulted in increased levels of GM-CSF secreted into the culture supernatant (149.32±3.76 pg/ml versus 123.27±4.05 pg/ml, n=3, Student *t* test, *p<0.05).

to that of the control group, representing an 18% and 20% increase, respectively (both Student *t* test, p<0.05, Figure 4).

DISCUSSION

In this study, we showed that an elevated level of GM-CSF in the vitreous is associated with the at-risk variant of the *CFH* Y402H polymorphism in postmortem human eyes. Several previous studies suggested that the *CFH* Y402H SNP is associated with a state of inflammation characterized by increased proinflammatory molecules systemically in the circulation system and locally in eye tissue [14,16,37,38]. Our findings are consistent with this idea, and extend the findings to include the vitreous, an important compartment of the eye. GM-CSF promotes the survival and activation of phagocytes, such as macrophages and microglia [23,24]. Our results also demonstrated more CD68-positive cells in eye tissues from donors with the at-risk CC variant. The higher levels of GM-CSF may promote or support these immune cells in the local retinal tissues of eyes genotyped with the at-risk CC variant in the *CFH gene*.

Although we observed immunoreactivity for CD68 in the choroid, immunoreactivity was at background levels in the neuroretina and the subretinal space, compartments that also contain microglia and macrophages. The accumulation of subretinal macrophages and neuroretina microglia might not be a characteristic of normal human eyes, as studied here, but of diseased eyes [39,40]. The change in the choroidal macrophages observed in our study is consistent with the trend shown in transgenic mice with the human at-risk CC variant [22].

The relationship between vitreal GM-CSF and the *CFH* Y402H polymorphism reported here supports the association between ocular proinflammatory mechanisms and the *CFH* genotype in the postmortem human eye. A limitation of this study is the relatively small sample size of genotyped tissues. Nevertheless, the in vitro stimulation results support our proposed hypotheses that GM-CSF synthesis is regulated in the RPE. We demonstrated the *GM-CSF gene* was upregulated in the RPE after stimulation with complement activation products, C3a and C5a. A non-polarized RPE cell model was used in this study, which might be of some limitation. Future studies using a polarized RPE culture model would be important to confirm and extend the findings of this study. Earlier studies showed that the *CFH* Y402H polymorphism is associated with an increased level of complement activation, and accordingly, its activation products in the eye [14,36,41,42]. Increased transcription of GM-CSF in the RPE stimulated by activation products, C3a and C5a, reported here and by others, suggests a possible mechanism by which GM-CSF levels are elevated in eyes with the at-risk CC variant [43]. However, since our results are from normal, non-diseased eye tissues, future studies on genotyped eyes with AMD are needed to extend these findings to the AMD eye. Earlier studies have shown that the levels of complement activation products in blood were especially higher in AMD cases compared to controls with the same genotype [36,44-47]. In addition, the secretion of GM-CSF was also elevated in RPE cells when stimulated by the cytokine TNF-α and the pro-oxidant HNE. Since the CC at-risk variant in the *CFH gene* was shown to promote oxidative stress and heighten cytokine levels, these factors may represent candidate mechanisms that underlie the elevated vitreal GM-CSF and choroidal macrophages seen in eyes with the CC at-risk variant in this study [16,17].

Conclusion: To our knowledge, this is the first study to report increased levels of GM-CSF in the vitreous and macrophages in the choroid of human donor eyes in association with the at-risk CC variant in the *CFH* Y402H SNP. Our results suggested that the polymorphism in the *CFH gene*, a known genetic risk factor for AMD, may contribute to the disease process through mechanisms that increase GM-CSF, a hematopoietic cytokine that promotes activation of macrophages and microglia. Further studies are required to evaluate the novel role of this cytokine in the development of chronic inflammatory retinal diseases such as AMD.

APPENDIX 1. THE GM-CSF LEVEL IN VITREOUS IS NOT ASSOCIATED WITH THE COLLECTION TIME.

Each point represented data from one donor sample. There is no relationship between the GM-CSF level and the collection time (Pearson correlation, n=20, r=0.2769, p=0.237). To access the data, click or select the words "Appendix 1."

ACKNOWLEDGMENTS

The authors thank and acknowledge Meysam Abbasi from Bio-Rad for technical assistance, and support by Canadian Institute of Health Research (CIHR) grant (MOP-97806 and MOP-126195 to Dr. Joanne Matsubara). The authors have no proprietary or commercial interest in any materials discussed in this article. Part of this work was presented at 2013 ARVO and Canadian Ophthalmological Society annual meeting poster sessions.

REFERENCES

1. Congdon N, O'Colmain B, Klaver CC, Klein R, Munoz B, Friedman DS, Kempen J, Taylor HR, Mitchell P. Eye Diseases Prevalence Research G. Causes and prevalence of visual impairment among adults in the United States. Arch Ophthalmol 2004; 122:477-85. [PMID: 15078664].
2. Jager RD, Mieler WF, Miller JW. Age-related macular degeneration. N Engl J Med 2008; 358:2606-17. [PMID: 18550876].
3. Khandhadia S, Cherry J, Lotery AJ. Age-related macular degeneration. Adv Exp Med Biol 2012; 724:15-36. [PMID: 22411231].
4. Pascolini D, Mariotti SP, Pokharel GP, Pararajasegaram R, Etya'ale D, Negrel AD, Resnikoff S. 2002 global update of available data on visual impairment: a compilation of population-based prevalence studies. Ophthalmic Epidemiol 2004; 11:67-115. [PMID: 15255026].
5. Kaarniranta K, Salminen A, Haapasalo A, Soininen H, Hiltunen M. Age-related macular degeneration (AMD): Alzheimer's disease in the eye? J Alzheimers Dis 2011; 24:615-31. [PMID: 21297256].
6. Ambati J, Atkinson JP, Gelfand BD. Immunology of age-related macular degeneration. Nat Rev Immunol 2013; 13:438-51. [PMID: 23702979].
7. Beatty S, Koh H, Phil M, Henson D, Boulton M. The role of oxidative stress in the pathogenesis of age-related macular degeneration. Surv Ophthalmol 2000; 45:115-34. [PMID: 11033038].
8. Swaroop A, Chew EY, Rickman CB, Abecasis GR. Unraveling a multifactorial late-onset disease: from genetic susceptibility to disease mechanisms for age-related macular degeneration. Annu Rev Genomics Hum Genet 2009; 10:19-43. [PMID: 19405847].
9. Edwards AO, Ritter R 3rd, Abel KJ, Manning A, Panhuysen C, Farrer LA. Complement factor H polymorphism and age-related macular degeneration. Science (New York, NY) 2005; 308:421-4. [PMID: 15761121].
10. Hageman GS, Anderson DH, Johnson LV, Hancox LS, Taiber AJ, Hardisty LI, Hageman JL, Stockman HA, Borchardt JD, Gehrs KM, Smith RJ, Silvestri G, Russell SR, Klaver CC, Barbazetto I, Chang S, Yannuzzi LA, Barile GR, Merriam JC, Smith RT, Olsh AK, Bergeron J, Zernant J, Merriam JE, Gold B, Dean M, Allikmets R. A common haplotype in the complement regulatory gene factor H (HF1/CFH) predisposes individuals to age-related macular degeneration. Proc Natl Acad Sci USA 2005; 102:7227-32. [PMID: 15870199].
11. Klein RJ, Zeiss C, Chew EY, Tsai JY, Sackler RS, Haynes C, Henning AK, SanGiovanni JP, Mane SM, Mayne ST, Bracken MB, Ferris FL, Ott J, Barnstable C, Hoh J. Complement factor H polymorphism in age-related macular degeneration. Science (New York, NY) 2005; 308:385-9. [PMID: 15761122].
12. Haines JL, Hauser MA, Schmidt S, Scott WK, Olson LM, Gallins P, Spencer KL, Kwan SY, Noureddine M, Gilbert JR, Schnetz-Boutaud N, Agarwal A, Postel EA, Pericak-Vance MA. Complement factor H variant increases the risk of age-related macular degeneration. Science (New York, NY) 2005; 308:419-21. [PMID: 15761120].
13. Johnson PT, Betts KE, Radeke MJ, Hageman GS, Anderson DH, Johnson LV. Individuals homozygous for the age-related macular degeneration risk-conferring variant of complement factor H have elevated levels of CRP in the choroid. Proc Natl Acad Sci USA 2006; 103:17456-61. [PMID: 17079491].
14. Mullins RF, Dewald AD, Streb LM, Wang K, Kuehn MH, Stone EM. Elevated membrane attack complex in human choroid with high risk complement factor H genotypes. Exp Eye Res 2011; 93:565-7. [PMID: 21729696].
15. Shaw PX, Zhang L, Zhang M, Du H, Zhao L, Lee C, Grob S, Lim SL, Hughes G, Lee J, Bedell M, Nelson MH, Lu F, Krupa M, Luo J, Ouyang H, Tu Z, Su Z, Zhu J, Wei X, Feng Z, Duan Y, Yang Z, Ferreyra H, Bartsch DU, Kozak I, Zhang L, Lin F, Sun H, Feng H, Zhang K. Complement factor H genotypes impact risk of age-related macular degeneration by interaction with oxidized phospholipids. Proc Natl Acad Sci USA 2012; 109:13757-62. [PMID: 22875704].
16. Cao S, Ko A, Partanen M, Pakzad-Vaezi K, Merkur AB, Albiani DA, Kirker AW, Wang A, Cui JZ, Forooghian F, Matsubara JA. Relationship between systemic cytokines and complement factor H Y402H polymorphism in patients with dry age-related macular degeneration. Am J Ophthalmol 2013; 156:1176-83. [PMID: 24083687].
17. Brantley MA Jr, Osborn MP, Sanders BJ, Rezaei KA, Lu P, Li C, Milne GL, Cai J, Sternberg P Jr. Plasma Biomarkers of Oxidative Stress and Genetic Variants in Age-Related Macular Degeneration. Am J Ophthalmol 2012; [PMID: 22035603].
18. Hamilton JA. Colony-stimulating factors in inflammation and autoimmunity. Nat Rev Immunol 2008; 8:533-44. [PMID: 18551128].

19. Grunin M, Hagbi-Levi S, Chowers I. The role of monocytes and macrophages in age-related macular degeneration. Adv Exp Med Biol 2014; 801:199-205. [PMID: 24664699].

20. Bao L, Haas M, Quigg RJ. Complement factor H deficiency accelerates development of lupus nephritis. Journal of the American Society of Nephrology: JASN 2011; 22:285-95. [PMID: 21148254].

21. Rowan S, Weikel K, Chang ML, Nagel BA, Thinschmidt JS, Carey A, Grant MB, Fliesler SJ, Smith D, Taylor A. Cfh genotype interacts with dietary glycemic index to modulate age-related macular degeneration-like features in mice. Invest Ophthalmol Vis Sci 2014; 55:492-501. [PMID: 24370827].

22. Ufret-Vincenty RL, Aredo B, Liu X, McMahon A, Chen PW, Sun H, Niederkorn JY, Kedzierski W. Transgenic mice expressing variants of complement factor H develop AMD-like retinal findings. Invest Ophthalmol Vis Sci 2010; 51:5878-87. [PMID: 20538999].

23. Lee SC, Liu W, Brosnan CF, Dickson DW. GM-CSF promotes proliferation of human fetal and adult microglia in primary cultures. Glia 1994; 12:309-18. [PMID: 7890333].

24. Ruef C, Coleman DL. Granulocyte-macrophage colony-stimulating factor: pleiotropic cytokine with potential clinical usefulness. Rev Infect Dis 1990; 12:41-62. [PMID: 2405468].

25. Jashnani KD, Kale SA, Rupani AB. Vitreous humor: biochemical constituents in estimation of postmortem interval. J Forensic Sci 2010; 55:1523-7. [PMID: 20666922].

26. Maberley D, Cui JZ, Matsubara JA. Vitreous leptin levels in retinal disease. Eye (Lond) 2006; 20:801-4. [PMID: 16052255].

27. Osuna E, Vivero G, Conejero J, Abenza JM, Martinez P, Luna A, Perez-Carceles MD. Postmortem vitreous humor beta-hydroxybutyrate: its utility for the postmortem interpretation of diabetes mellitus. Forensic Sci Int 2005; 153:189-95. [PMID: 16139109].

28. Wang JCC, Wang AK, Gao JY, Cao SJ, Samad I, Zhang DA, Ritland C, Cui JZ, Matsubara JA. Technical Brief: Isolation of total DNA from postmortem human eye tissues and quality comparison between iris and retina. Mol Vis 2012; 18:3049-56. [PMID: 23288996].

29. Kurji KH, Cui JZ, Lin T, Harriman D, Prasad SS, Kojic L, Matsubara JA. Microarray analysis identifies changes in inflammatory gene expression in response to amyloid-beta stimulation of cultured human retinal pigment epithelial cells. Invest Ophthalmol Vis Sci 2010; 51:1151-63. [PMID: 19797223].

30. Cao S, Walker GB, Wang X, Cui JZ, Matsubara JA. Altered cytokine profiles of human retinal pigment epithelium: Oxidant injury and replicative senescence. Mol Vis 2013; 19:718-28. [PMID: 23559866].

31. Seth A, Cui J, To E, Kwee M, Matsubara J. Complement-associated deposits in the human retina. Invest Ophthalmol Vis Sci 2008; 49:743-50. [PMID: 18235023].

32. Fischer HG, Bielinsky AK, Nitzgen B, Daubener W, Hadding U. Functional dichotomy of mouse microglia developed in vitro: differential effects of macrophage and granulocyte/macrophage colony-stimulating factor on cytokine secretion and antitoxoplasmic activity. J Neuroimmunol 1993; 45:193-201. [PMID: 8331161].

33. Murray PJ, Wynn TA. Protective and pathogenic functions of macrophage subsets. Nat Rev Immunol 2011; 11:723-37. [PMID: 21997792].

34. Cherepanoff S, McMenamin P, Gillies MC, Kettle E, Sarks SH. Bruch's membrane and choroidal macrophages in early and advanced age-related macular degeneration. Br J Ophthalmol 2010; 94:918-25. [PMID: 19965817].

35. Planck SR, Huang XN, Robertson JE, Rosenbaum JT. Retinal pigment epithelial cells produce interleukin-1 beta and granulocyte-macrophage colony-stimulating factor in response to interleukin-1 alpha. Curr Eye Res 1993; 12:205-12. [PMID: 8482109].

36. Scholl HP, Charbel Issa P, Walier M, Janzer S, Pollok-Kopp B, Borncke F, Fritsche LG, Chong NV, Fimmers R, Wienker T, Holz FG, Weber BH, Oppermann M. Systemic complement activation in age-related macular degeneration. PLoS ONE 2008; 3:e2593-[PMID: 18596911].

37. Liu B, Wei L, Meyerle C, Tuo J, Sen HN, Li Z, Chakrabarty S, Agron E, Chan CC, Klein ML, Chew E, Ferris F, Nussenblatt RB. Complement component C5a promotes expression of IL-22 and IL-17 from human T cells and its implication in age-related macular degeneration. J Transl Med 2011; 9:1-12. [PMID: 21762495].

38. Mooijaart SP, Koeijvoets KM, Sijbrands EJ, Daha MR, Westendorp RG. Complement Factor H polymorphism Y402H associates with inflammation, visual acuity, and cardiovascular mortality in the elderly population at large. Exp Gerontol 2007; 42:1116-22. [PMID: 17869048].

39. Gupta N, Brown KE, Milam AH. Activated microglia in human retinitis pigmentosa, late-onset retinal degeneration, and age-related macular degeneration. Exp Eye Res 2003; 76:463-71. [PMID: 12634111].

40. Zeng HY, Green WR, Tso MO. Microglial activation in human diabetic retinopathy. Arch Ophthalmol 2008; 126:227-32. [PMID: 18268214].

41. Anderson DH, Radeke MJ, Gallo NB, Chapin EA, Johnson PT, Curletti CR, Hancox LS, Hu J, Ebright JN, Malek G, Hauser MA, Rickman CB, Bok D, Hageman GS, Johnson LV. The pivotal role of the complement system in aging and age-related macular degeneration: hypothesis re-visited. Prog Retin Eye Res 2010; 29:95-112. [PMID: 19961953].

42. Loyet KM, Deforge LE, Katschke KJ Jr, Diehl L, Graham RR, Pao L, Sturgeon L, Lewin-Koh SC, Hollyfield JG, van Lookeren Campagne M. Activation of the alternative complement pathway in vitreous is controlled by genetics in age-related macular degeneration. Invest Ophthalmol Vis Sci 2012; 53:6628-37. [PMID: 22930722].

43. Fukuoka Y, Strainic M, Medof ME. Differential cytokine expression of human retinal pigment epithelial cells in

response to stimulation by C5a. Clin Exp Immunol 2003; 131:248-53. [PMID: 12562384].

44. Reynolds R, Hartnett ME, Atkinson JP, Giclas PC, Rosner B, Seddon JM. Plasma complement components and activation fragments: associations with age-related macular degeneration genotypes and phenotypes. Invest Ophthalmol Vis Sci 2009; 50:5818-27. [PMID: 19661236].

45. Hecker LA, Edwards AO, Ryu E, Tosakulwong N, Baratz KH, Brown WL, Charbel Issa P, Scholl HP, Pollok-Kopp B, Schmid-Kubista KE, Bailey KR, Oppermann M. Genetic control of the alternative pathway of complement in humans and age-related macular degeneration. Hum Mol Genet 2010; 19:209-15. [PMID: 19825847].

46. Smailhodzic D, Klaver CC, Klevering BJ, Boon CJ, Groenewoud JM, Kirchhof B, Daha MR, den Hollander AI, Hoyng CB. Risk alleles in CFH and ARMS2 are independently associated with systemic complement activation in age-related macular degeneration. Ophthalmology 2012; 119:339-46. [PMID: 22133792].

47. Ristau T, Paun C, Ersoy L, Hahn M, Lechanteur Y, Hoyng C, de Jong EK, Daha MR, Kirchhof B, den Hollander AI, Fauser S. Impact of the common genetic associations of age-related macular degeneration upon systemic complement component C3d levels. PLoS ONE 2014; 9:e93459-[PMID: 24675670].

Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 13 March 2015. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.