**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-a 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.
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 GTA 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^ΔΔCt 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 NaH2PO4, 1.8 mM KH2PO4, pH 7.4). Then, 200 µl of 1X 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% H2O2 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 mm². 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 of eyes with the at-risk homozygous CC variant in 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,
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 mm², n=4) compared to the TT eyes (1.12±0.20 per 0.1 mm², 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].

### 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%)           |

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).

### 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      |         |
| GM-CSF (pg/ml) | Mean 607.54 85.83       | Mean 656.32 15.20 | Mean 286.69 81.96 | 0.0003 |
| Age (years)    | Mean 60.25 6.84         | Mean 58.90 4.48 | Mean 62.50 4.22 | 0.8505 |
| Collection time (h) | Mean 14.50 1.76 | Mean 16.56 1.58 | Mean 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 mm² 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 mm², \( n=4 \)) compared to the TT eyes (1.12±0.20 per 0.1 mm², \( n=6 \); 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.”

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