Cataract Surgery Induces Retinal Pro-inflammatory Gene Expression and Protein Secretion

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PURPOSE. To investigate the effect(s) of cataract surgery on the expression of pro-inflammatory genes and proteins in the retina using an experimental rodent model.

METHODS. An extracapsular lens extraction was performed in one eye of C57BL/6 mice (n = 24); the contralateral unoperated eyes (n = 24) as well as eyes from unoperated animals (n = 9) served as controls. The neurosensory retina and retinal pigment epithelium (RPE)/choroid were collected postoperatively. Expression of genes involved in the acute inflammatory/injury response, including IL-1β, fibroblast growth factor, transforming growth factor β, chemokine CCL2, SDF-1, and complements C3, C4, and factor B (CFB), were examined by real-time PCR and, selectively, by immunohistochemistry.

RESULTS. The expression of IL-1β and CCL2 genes was markedly upregulated (≥20-fold, P < 0.01) in the neurosensory retina 30 minutes postoperatively and maintained for the 2-week postoperative period of observation; IL-1β expression was also upregulated in RPE/choroid. The expression of complement C3 (>5-fold) and CFB (>30-fold) genes in the neurosensory retina was also significantly upregulated (P < 0.01 in both cases). Increased IL-1β, CCL2, and CFB as well as enhanced C5b-9 immunostaining were observed by confocal microscopy.

CONCLUSIONS. In rodents, lens extraction elicited an acute pro-inflammatory gene and protein response in the posterior segment of the eye, indicating induction of the inflammasome as well as complement activation, as occurs in the “danger” response. A similar response in humans might explain the pathogenesis of cataract surgery–associated retinal complications such as cystoid macular edema. (Invest Ophthalmol Vis Sci. 2011;52:249–255) DOI:10.1167/iovs.10-6001

Cataract, the opacification of the crystalline lens of the eye, is the leading cause of blindness worldwide. Cataract surgery represents the commonest ophthalmic surgical procedure performed in eye clinics; in the United States approximately 1.3 million cataract operations are undertaken each year (http://www.nei.nih.gov/resources/strategicplans/neiplan/frm_lens.asp). Although cataract surgery is most often associated with restoration of vision, in several instances this intervention may lead to worsening of preexisting retinal disease and visual loss. Several studies have suggested that cataract surgery may aggravate diabetic retinopathy and maculopathy1–6 and age-related macular degeneration,7–12 although the latter remains under debate.11,13 Furthermore, cataract surgery may lead to the development of retinal disease in the form of pseudophakic macular edema.14,15

Very scarce experimental data exist regarding the possible effects of cataract surgery in the retina. Tso and Shih16 evaluated clinical, light, and electron microscopic changes taking place at the macula in seven rhesus monkeys after lens extraction. No evidence of breakage of the blood–retinal barrier (BRB), as determined by fluorescein angiography, was observed when surgery was not complicated with vitreous loss; however, in these cases, the macula appeared mildly thickened.16 Disruption of the outer BRB (i.e., the tight junctions between retinal pigment epithelium [RPE]) was observed on light and electron microscopy in all eyes. In a mouse study, however, Liu and colleagues17 observed breakdown of the inner BRB (i.e., the tight junctions between the endothelia of retinal blood vessels) after incomplete aspiration of the lens.

The explanation for these retinal changes after cataract surgery is unclear. For instance, it has been variably suggested that inflammatory mediators, prostaglandin release, and alteration of tissue architecture with mechanical changes might play a part in these complications. The purpose of the present study was, thus, to investigate the effect(s) of lens extraction on pro-inflammatory gene expression and protein secretion in retinal/choroidal tissues of the eye using a mouse model. Specifically, we used immunocytochemistry and real-time PCR to evaluate the effect of this surgical procedure on the expression of genes and their products involved in the inflammatory response and in the response to injury ("danger") in the neurosensory retina and RPE/choroid.

MATERIALS AND METHODS

Animals

C57BL/6 male mice (3 months old) were used in this study. All mice were housed and bred in a normal experimental room and exposed to a 12-hour dark–12-hour light cycle. All procedures concerning the use of animals in this study were performed according to the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research and under the regulations of the UK Animal License Act 1986.

Experimental Design

Lens extraction was performed in 24 mice. Initially, 12 mice underwent lens extraction in the right eye (Surgery eye [S_eye]), the left eye remained unoperated and was used as an internal control (Fellow eye [F_eye]). Nine unoperated mice were used as normal mouse controls (unoperated control [U_C]). Two weeks after surgery, six mice from each group were killed, and eyes were collected for gene expression (quantitative real-time RT-PCR [qPCR]). The remaining mice (six oper-
ated mice and three unoperated control mice) were killed at 4 weeks after surgery, and eyes were collected for immunohistochemistry.

Based on data from the initial study, a time course study examining selected genes and proteins was performed using a further 12 mice, who underwent lens extraction in the right eye (S_eye) while the left eye was used as an internal control (F_eye). Three mice were killed at each time point (30 minutes, 24 hours, 48 hours, and 1 week) post-surgery, and the tissues harvested for gene expression (qPCR).

Lens Extraction

An extracapsular lens extraction was performed in the right eye of 24 C57BL/6 mice; surgeries were done consecutively. Animals were anesthetized using intrapitoneal ketamine hydrochloride (Fort George Animal Centre, Southampton, UK) at a dose of 60 mg/kg and xylazine (Pharmacia & Veterinary Products, Kiel, Germany) at a dose of 5 mg/kg. Pupillary dilation was achieved by using 1% tropicamide and 2.5% phenylephrine (Chauvin, Essex, England). The surgery was performed as described previously.18,19 Briefly, a corneal incision, of ~100–120°, was made, and 1% sodium hyaluronate (Microvisc; Bohus BioTech, Bjorko, Sweden) was inserted in the anterior chamber (AC). The corneal incision was then extended, and an anterior curvilinear continuous capsulorhexis was performed followed by hydrodissection and lens removal. The AC was filled with 1% sodium hyaluronate, and the corneal wound was closed using interrupted sutures. Topical 2.5% phenylephrine, 1% tropicamide, and 1% atropine (all from Chauvin) were administered at the end of the surgery.

Sample Collection for RNA Extraction

At different times after surgery (30 minutes, 24 hours, 48 hours, 1 week, 2 weeks), mice were killed by CO2 inhalation. Eyes were then carefully removed. For RNA extraction, immediately after enucleation, eyes were transferred onto a Petri dish containing ice-cold PBS. The neurosensory retina and RPE/choroidal tissue were dissected under a microscope (LSM510 META; Carl Zeiss Meditc, Göttingen, Germany). Total RNA was isolated (RNeasy Mini Kit; Qiagen, West Sussex, UK) and then reverse transcribed into cDNA (SuperScript II reverse transcriptase; Invitrogen, Paisley, UK). All procedures were conducted following the manufacturers’ instructions. The same amount of cDNA was used as a template for real-time PCR. All real-time PCRs were performed in 96-well plates (LightCycler 480 with LightCycler 480 Probes Master; Roche Diagnostics, CITY, Germany) and relevant primers and probes. For C3 and C4 genes, a hot-start PCR reaction mix was performed as described previously.18,19

qPCR

Total RNA was isolated (RNeasy Mini Kit; Qiagen, West Sussex, UK) and then reverse transcribed into cDNA (SuperScript II reverse transcriptase; Invitrogen, Paisley, UK). All procedures were conducted following the manufacturers’ instructions. The same amount of cDNA was used as a template for real-time PCR. All real-time PCRs were performed in 96-well plates (LightCycler 480 with LightCycler 480 Probes Master; Roche Diagnostics, CITY, Germany) and relevant primers and probes. For C3 and C4 genes, a hot-start PCR reaction mix was used (LightCycler 480 SYBR Green Probes Master; Roche Diagnostics). Primers were designed using a web assay design center (Roche Applied Science, Mannheim, Germany; www.universalprobelibrary.com). All primers were ordered from Sigma-Aldrich (Sigma-Aldrich, Dorset, UK). FAM-labeled probes (Universal Probe Library) were ordered from Roche Applied Science. The sequence of primers and probe numbers used in this study is listed in Table 1. For genes encoding CCL2 and IL1β, a gene expression assay was used (CCL2: Mm99999056_m1, IL1β: 01336189_m1; TaqMan Gene Expression Assay; Applied Biosystems, Warrington, UK). Mouse GAPDH conjugated with a fluorescence dye (VIC; Applied Biosystems) was used as the reference gene. Gene expression levels were first normalized to the GAPDH mRNA level, and the normalized expression levels were then calculated as fold changes of their controls.

Immunohistochemistry

Mouse eyes were embedded in optimum cutting temperature compound (OCT) and stored at −30°C. Cryosections of mouse eyes were fixed with 2% paraformaldehyde (Agar Scientific, Cambridge, UK) for 15 minutes at room temperature. After blocking with 10% BSA, samples were incubated with a primary antibody (1:100 dilution with 1% BSA-PBS) for a further hour. Samples were washed and mounted in medium with propidium iodide or 4’,6-diamidino-2-phenylindole dihydrochloride (Vectashield Mounting Medium; Vector Laboratories, Peterborough, UK). The primary antibodies used in the study include goat anti-mouse IL1β polyclonal antibody, rabbit anti-mouse CCL2 polyclonal antibody, goat anti-human complement factor B (CfB) polyclonal antibody (all antibodies from Abcam, Cambridge, UK), and biotinylated anti-mouse C3d (R&D Systems, Minneapolis, MN). The secondary antibodies include fluoroscein isothiocyanate (FITC) conjugated anti-goat IgG, R-PE conjugated streptavidin (1:200, both from BD Biosciences, Oxford, UK), and FITC conjugated anti-rabbit IgG (Invitrogen). All samples were examined with a confocal microscope (LSM510 META; Carl Zeiss Meditec, Göttingen, Germany).

Data Analysis

Gene fold changes of operated eyes versus unoperated fellow eyes were compared using a paired Student’s t-test. Gene fold changes between eyes of mice undergoing surgery (including operated and unoperated eyes) and normal unoperated mice were compared using a one-way Tukey’s ANOVA multiple comparison test. Probability values of P < 0.05 were considered statistically significant. Data were expressed as mean ± SE.

RESULTS

Cytokine/Chemokine Expression/Production in Neuroretina, RPE/Choroid after Lens Extraction

Gene Expression. To explore the mechanism of cataract surgery–related retinal complications such as macular edema, we first investigated the expression of genes that might affect the BRB, including cytokine IL-1β, chemokines CCL2 and SDF-1, and growth factors FGF and VEGF in the retina and RPE/choroid. Two weeks after surgery, the expression of IL-1β, CCL2, and FGF genes was statistically significantly upregulated in the neurosensory retina of mice that had cataract extraction (S_eye) when compared with fellow unoperated eyes (internal control, F_eye; P < 0.01) as well as with eyes

Table 1. Primer Sequences

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<th>Gene Name</th>
<th>Left Primer</th>
<th>Right Primer</th>
<th>UPL No.</th>
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<tr>
<td>VEGF</td>
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<td>GGGTTACGAGTTCCCGGAA</td>
<td>4</td>
</tr>
<tr>
<td>SDF-1a</td>
<td>CAGTGGCCTCTGTGACGACTG</td>
<td>TAATTTCCGTCCTAAATGACGC</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>C3</td>
<td>AGCAGCAGTACATCGAGGCC</td>
<td>GATGATAGCTGATGTGTTG</td>
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UPL, Universal Probe Library (Roche Applied Science).
from normal, unoperated mice (U-C_eyes, P < 0.01; Fig. 1A). Interestingly, the expression of IL-1β in the neurosensory retina of S_eyes was also statistically significantly increased (>5-fold, P < 0.01) when compared with that of U-C_eyes (Fig. 1A). A small but statistically significant increase in gene expression of SDF-1 was also observed in the neurosensory retina of S_eyes when compared with U-C_eyes (2.14-fold, P < 0.05; Fig. 1A). Importantly, VEGF gene expression was not affected by lens extraction (Fig. 1A).

In the RPE/choroid, the expression of IL-1β was significantly upregulated in both S_eyes (10-fold, P < 0.01) and F_eyes (4.7-fold, P < 0.01) when compared with U-C_eyes (Fig. 1B). No statistically significant differences in the expression levels of other genes, including CCL2, SDF-1, and VEGF, were detected between S_eyes and F_eyes (Fig. 1B).

To understand how soon the surgery might affect retina and RPE/choroidal gene expression, we then carried out a time course study. Thirty minutes after lens extraction, the expression of IL-1β and CCL2 genes was massively upregulated (>20-fold) in the neuroretina of S_eyes compared with F_eyes (Fig. 2A). By 24 hours after surgery, both genes increased over 50-fold (Fig. 2A) and remained at high levels at 1 week postsurgery (Fig. 2A). The expression of TGFβ and FGF genes was also statistically significantly upregulated at 48 hours and 1 week postoperatively (Fig. 2A). The expression of VEGF and SDF-1, although appearing to be elevated, was not statistically significantly increased (Fig. 2A).

In RPE/choroid, the expression of CCL2 and IL-1β genes was statistically significantly increased in S_eyes compared with unoperated F_eyes at 24 hours postoperation (Fig. 2B). However, the level of upregulation (between 10~20-fold; Fig. 2B) was less marked than that in the retina (between 20~50-fold; Fig. 2A). The expression of TGF-β and SDF-1 was statistically significantly increased at 24 hours postsurgery (Fig. 2B).

Protein Expression. Having shown that the transcriptions of IL-β and CCL2 genes are massively upregulated after lens extraction, particularly in the neuroretina, we then examined their protein expression. IL-1β was not detected in the retina, choroid, or sclera of eyes of unoperated mice (Figs. 3A, 3D), but strong immunostaining was observed at the ganglion cell layer, inner nuclear layer, and choroid of S_eyes (Figs. 3C, 3F). Interestingly, discrete IL-1β positivity was also detected in the ganglion cell layer and choroid of F_eyes (Figs. 3B, 3E).

In U-C_mice, irregular patches of CCL2 were detected in the retina, RPE, and choroid (Figs. 4A, 4D). Four weeks after
lens extraction, the expression of CCL2 was significantly increased in S\textsubscript{eyes} (Figs. 4C, 4F) but not in F\textsubscript{eyes} (Figs. 4B, 4E).

**Effect of Lens Extraction on Neurosensory Retina, RPE/Choroid Complement Activation**

We have shown previously that a local complement regulatory system exists in the retina and RPE/choroidal tissue.\textsuperscript{20,21} To understand whether lens extraction could elicit local complement activation, we investigated the complement gene/protein expression in eyes undergoing lens extraction. Because cytokine/chemokine changes were predominately observed in the retina (Figs. 1 and 2), we focused our investigation on retinal tissue. An statistically significant increased gene expression of C3 and CFB (5.35-fold and 31.46-fold increase, respectively, \(P < 0.01\) for both) was observed in the neurosensory retina after lens extraction (S\textsubscript{eyes}) when compared with that observed in eyes of mice that did not undergo surgery (F\_eyes and U-C\_eyes) (Fig. 5A). The expression of the C4 gene was not significantly affected by the surgery (Fig. 5A).

Similar to our previous observations,\textsuperscript{20,21} a low level of CFB staining at the apical area of RPE cells (Fig. 5B, arrows) and C3d deposition (Fig. 5B, arrowheads) in Bruch’s membrane were detected in F\_eyes and U-C\_eyes (Fig. 5B). Four weeks after lens extraction, the expression of CFB and C3d was significantly increased in S\_eyes (Fig. 5C). Furthermore, CFB was also detected in the neurosensory retina of S\_eyes (Fig. 5C, asterisks). To further understand whether increased C3 and CFB gene expressions resulted in a full activation of the complement cascade, we examined the terminal complement activation product C5b-9 (membrane attack complex [MAC]). Immunostaining for MAC was negligible in the posterior segment of eyes of U-C\_mice as well as in F\_eyes (Fig. 5E). In contrast, in S\_eyes, MAC expression was observed throughout the neurosensory retina (Fig. 5F).

**DISCUSSION**

Here we showed that, in mice, lens extraction induces the acute, if not instant, expression of pro-inflammatory genes in the posterior segment of the eye, predominately in the neurosensory retina. The inflammatory/immune activation is manifested by the massive production of inflammatory cytokine IL-1\textsubscript{beta} and chemokine CCL2 and the activation of the complement cascade. Complement activation appeared to be mediated through the alternative pathway because CFB, but not C4, gene expression was upregulated (>31-fold) after lens extraction.\textsuperscript{22} The induction of IL-1\textsubscript{beta} is particularly interesting because this IL-1\textsubscript{beta} is normally generated after activation of the “inflammasome” through stimulation of receptors involved in “danger” responses, involving a series of signaling molecules and numerous potentially different pathways (for review see Ref. 23). It will be of interest to identify which of these signaling pathways is involved in retinal tissues after cataract extraction.
How lens extraction induces retinal inflammation is not known. We have previously shown that, in rodents, lens extraction induces a low-grade inflammatory reaction in the anterior segment of the eye, which is maintained for at least 2 weeks after surgery. This low-grade inflammation seems to play a role in the occurrence of the posterior segment response in the anterior segment of the eye. The fact that lens extraction induces acute retinal immune/inflammation/cytokine gene activation may have important implications in cataract surgery–associated retinal complications, including pseudophakic macular edema, and especially so if there is coexisting pathology such as occurs in the progression of diabetic retinopathy/maculopathy and neovascular AMD, both of which are observed after this surgical procedure. Key events on the above mentioned retinal complications are the breakdown of the inner and outer BRB and the growth of abnormal new blood vessels. IL-1β is an important mediator of the inflammatory response and its gene expression has been shown to be a potent inducer of BRB breakdown. A single dose of intravitreally administered recombinant IL-1β induces a rapid (3–4 hours) BRB breakdown and leukocyte infiltration. The BRB disruption may last for more than 2 days. Perivascular macrophages are important for retinal immune surveillance and for the maintenance of the BRB. IL-1β can induce retinal perivascular macrophage activation and vascular disassociation. Further, IL-1β appears to be involved in the disruption of BRB in diabetic retinopathy. IL-1β might also affect retinal angiogenesis. Mice with IL-1β overexpression have increased retinal VEGF expression. Although upregulation of VEGF gene was not observed in this study in which lens extraction was performed in eyes of normal, healthy mice, under different circumstances in which VEGF expression is already at pathologic levels such as in diabetic retinopathy and in AMD, the increased IL-1β could potentially alter further VEGF expression, promoting neovascularization.

Complement activation is believed to play an important role in the occurrence of AMD. Increased complement activity is also observed in diabetic retinopathy. In this study, we have found that lens extraction induces AP-mediated complement activation in the neurosensory retina (Fig. 5). The detailed mechanism(s) leading to retina complement activation remains elusive. Breakdown of the BRB might result in intraretinal leakage of a variety of plasma proteins, which may trigger complement activation. In addition, the inflammatory response may also induce local complement production. We have shown previously that RPE cells can produce CFB. Further studies have shown that the production CFB by RPE cells can be upregulated by inflammatory cytokines TNF-α and IFN-γ, and IL-1β. A more recent study has shown that a variety of complement components can be produced locally in the eye by the neural retina and the RPE/choroid. Retinal complement activation elicited by lens extraction may play critical roles in cataract surgery–related AMD progression. Inflammation is mediated by chemotactic responses in leukocytes. The chemokine CCL2 is especially important for monocyte chemotactic migration. CCL2 is expressed and produced constitutively by retinal microvascular endothelial cells and RPE cells. CCL2 and CCL5 are often coupled together, particularly during acute innate immune responses. IL-1β has been shown to be a potent inducer of CCL2 production in both retinal endothelial cells and RPE cells. Increased CCL2 production may therefore be responsible for IL-1β–mediated leukocyte infiltration. The patho-physiologic role of CCL2 in retinal diseases is not fully understood. Mice deficient in CCL2 have been reported to develop retinal changes similar to AMD. Whether the increased CCL2 expression observed here in eyes after lens extraction represents a tissue protective response (to restore tissue homeostasis) or a consequence of uncontrolled immune responses via "danger signals" such as the release of uric acid from dying or injured cells or the accumulation of insoluble protein aggregates will occur in the eye after lens extraction. Related work in the field of wound healing and trauma has identified interesting novel mechanisms for the induction of innate immune responses via "danger signals" such as the release of uric acid from dying or injured cells or the accumulation of insoluble protein aggregates.
response (which may cause pathology) warrants further investigation. In this study, lens extraction also upregulated IL-1β gene expression in unoperated eyes (Fig. 1). Systemic sensitization via the eye is clinically and experimentally well recognized, particularly in the context of sympathetic ophthalmia and induction of an adaptive autoimmune response.45–47 Systemic sensitization leading to sympathetic ophthalmia has been reported after cataract extraction but usually when the surgery was complicated.48–50 Autoimmune disease generally is currently considered to develop after “nonspecific” induction of the innate immune system, particularly involving components of the inflammasome.31,51 It is therefore possible that simple trauma or exposure to bacterial products such as lipopolysaccharide can set the scene for the occurrence of autoimmune disease. In this respect it is of interest that induction of endotoxin-induced uveitis by unilateral intravital injection of endotoxin is accompanied with hypopyon uveitis in the uninjected fellow eye.53 There is therefore a precedent for activation of an innate immune response in the fellow eye, which may be mirrored in the present study by the upregulation of inflammation-associated genes by surgery to the first eye. It has been speculated that inflammation may play a role in cataract surgery-mediated retinal complications. Corticosteroids, as well as nonsteroidal anti-inflammatory drugs, have been shown to be beneficial to patients with pseudophakic macular edema.54–56 Our study provides the first experimental evidence that lens extraction induces a pro-inflammatory response in the retina, which would partially explain the above effects. Furthermore, current findings, if demonstrated in experimental models of diabetic retinopathy and early AMD, could help in establishing a causative link between cataract surgery and diabetic retinopathy/maculopathy/AMD progression, as well as the development of treatment strategies for these complications. It should be acknowledged, however, that the pro-inflammatory response observed in the current mice model of extracapsular cataract extraction may not represent exactly that occurring in humans after small incision phacoemulsification and intraocular lens implantation.

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References


