Profile of Lipid and Protein Autacoids in Diabetic Vitreous Correlates With the Progression of Diabetic Retinopathy

Michal Laniado Schwartzman,[1,2] Pavel Iserovich,[3] Katherine Gotlinger,[1] Lars Bellner,[1] Michael W. Dunn,[2] Mauro Sartore,[4] Maria Grazia Pertile,[4] Andrea Leonardi,[5] Sonal Sathe,[3] Ann Beaton,[3] Lynn Trieu,[3] and Robert Sack[3]

OBJECTIVE—This study was aimed at obtaining a profile of lipids and proteins with a paracrine function in normal and diabetic vitreous and exploring whether the profile correlates with retinal pathology.

RESEARCH DESIGN AND METHODS—Vitreous was recovered from 47 individuals undergoing vitreoretinal surgery: 16 had nonproliferative diabetic retinopathy (NPDR), 15 had proliferative diabetic retinopathy, 7 had retinal detachments, and 9 had epiretinal membranes. Protein and lipid autacoid profiles were determined by protein arrays and mass spectrometry–based lipidomics.

RESULTS—Vitreous lipids included lipoxygenase (LO)- and cytochrome P450 epoxygenase (CYP)-derived eicosanoids. The most prominent LO-derived eicosanoid was 5-hydroxyeicosate traenoic acid (HETE), which demonstrated a diabetes-specific increase ($P = 0.027$) with the highest increase in NPDR vitreous. Vitreous also contained CYP-derived epoxyeicosatrienoic acids; their levels were higher in nondiabetic than diabetic vitreous ($P < 0.05$). Among inflammatory, angiogenic, and angiostatic cytokines and chemokines, only vascular endothelial growth factor (VEGF) showed a significant diabetes-specific profile ($P < 0.05$), although a similar trend was noted for tumor necrosis factor (TNF)-α. Soluble VEGF receptors R1 and R2 were detected in all samples with lowest VEGF-R2 levels ($P < 0.05$) and higher ratio of VEGF to its receptors in NPDR and PDR vitreous.

CONCLUSIONS—This study is the first to demonstrate diabetes-specific changes in vitreous lipid autacoids including arachidonate and docosahexanoate-derived metabolites indicating an increase in inflammatory versus anti-inflammatory lipid mediators that correlated with increased levels of inflammatory and angiogenic proteins, further supporting the notion that inflammation plays a role the pathogenesis of this disease. ***Diabetes*** **59:1780–1788, 2010**

From the [1]Department of Pharmacology, New York Medical College, Valhalla, New York; the [2]Department of Ophthalmology, New York Medical College, Valhalla, New York; the [3]Department of Biological Sciences, SUNY Optometry, New York, New York; [4]Ospedale Sacro Cuore, Negrar (VR), Italy; and the [5]Department of Neuroscience, University of Padua, Padua, Italy.

Corresponding author: Michal Laniado Schwartzman, michal_schwartzman@nymc.edu.

Received 22 January 2010 and 17 April 2010. Published ahead of print at http://diabetes.diabetesjournals.org on 27 April 2010. DOI: 10.2337/db10-0110.

The evolving concept that there is an inflammatory basis for diabetic retinopathy in its early stages characterized by overexpression of proinflammatory factors has gained much support and has led to the identification of potent proinflammatory transcription factors, chemokines, and cytokines in diabetic retinas and vitreous (1–3). There are two main stages of diabetic retinopathy—the earliest, nonproliferative diabetic retinopathy (NPDR), is characterized by structural changes in capillaries that lead to bleeding and leakage and now is thought to have an inflammatory basis. As the disease progresses, angiogenic factors are secreted that induce the growth of new retinal blood vessels (neovascularization), which marks the next and most destructive phase, proliferative diabetic retinopathy (PDR).

A noninclusive list of upregulated factors in the diabetic vitreous includes vascular endothelial growth factor (VEGF), VEGF angiogenic isoforms angiogenin (ANG), angiopoietin (ANG-2), hepatic growth factor (HGF), insulin like growth factor (IGF)-1, interleukins (IL-8, IL-6, IL-10), leptin, matrix metalloproteases (MMP-9, MMP-2), and monocyte chemoattractant protein-1 (MCP-1) among others. Also seen in the diabetic vitreous are parallel decreases in the concentrations of many angiostatic factors including pigment epithelial-derived factor (PEDF), endostatin, and the soluble vascular endothelial growth factor receptor-1 (VEGF-R1) (3–6). Many of these factors are multifunctional in nature. There is now considerable evidence that some of the angiogenic factors also act to increase nerve and other cellular apoptotic processes, thereby contributing to the compromised functional integrity of the neurological processing network in the retina and other tissues. To what extent specific factors contribute to the overall pathologic processes is currently uncertain, since comparable changes in the concentration of many of these factors have been observed secondary to retinal detachments that are not diabetic related.

Almost all the work exploring the presence of inflammatory and angiogenic molecules in the diabetic vitreous has focused on bioactive proteins and has ignored the contributions of lipid mediators including various arachidonic acid–derived eicosanoids of the cyclooxygenase (COX), lipoxygenase (LO), and cytochrome P450 monooxygenase (CYP) pathways. Although eicosanoids released from infiltrating cells can amplify the inflammatory response, their ability to be produced endogenously from the injured tissue renders them able to initiate the inflammatory response by altering vascular permeability and

stimulating leukocyte chemotaxis. Prominent proinflammatory and angiogenic eicosanoids include the COX-derived prostaglandins (PGE_2 and TxB_2), the LO-derived leukotrienes, and the CYP-derived 12(R)-hydroxyeicosatrienoic acid (12-HETrE) (7–9). Among the anti-inflammatory eicosanoids are the LO-derived lipoxins (10) and CYP-derived epoxyeicosatrienoic acids (EETs) (11). The role of eicosanoids in the pathogenesis of diabetic retinopathy is largely unknown.

The vitreous accumulates lipids and proteins with paracrine functions from the retina. This study aims at obtaining a partial profile of these entities in the normal and diabetic vitreous and exploring whether their presence correlates with retinal pathology. Accordingly, vitreous samples from nondiabetic patients with retinal detachment (RD) or from patients undergoing epiretinal membrane (ERM) surgery and from diabetic patients with PDR and NPDR were analyzed. This study is the first to demonstrate diabetes-specific changes in vitreous eicosanoids indicating an increase in inflammatory versus anti-inflammatory lipid mediators. It is also the first to document the presence of soluble VEGF-R2 in human vitreous and to suggest that the relative concentration of VEGF to its soluble receptors is indicative of the diabetic status.

RESEARCH DESIGN AND METHODS

Vitreous was obtained after informed consent according to the Association for Research in Vision and Ophthalmology guidelines and with the approval of institutional review boards from the University Hospital of Padua, Italy, and Sacro Cuore Hospital, Negrar VR, Italy. Vitreous samples were obtained from 47 patients undergoing vitreoretinal surgery: 31 (66 ± 2 years old) were diabetic, with fasting blood glucose at the time of surgery averaging 169.40 ± 12.71 mg/dl ($P < 0.02$ vs. nondiabetic subjects), 16 (68 ± 2 years old; 166.50 ± 24.89 mg glucose/dl) had NPDR, and 15 (63 ± 3 years old; 171.50 ± 13.20 mg glucose/dl) had PDR. All patients with NPDR or PDR were in the "active" form of the disease at the time of surgery and were diagnosed with either type 2 diabetes ($n = 28$) or type 1 diabetes ($n = 3$). The remaining 16 nondiabetic subjects (64 ± 4 years old) with blood glucose of 120.50 ± 12.26 mg/dl underwent vitreoretinal surgery for repair of a retinal detachment (RD) ($n = 7$; 55 ± 7 years old; 142.00 ± 26.33 mg glucose/dl) or surgery for an epiretinal membrane (ERM) ($n = 9$; 71 ± 2 years old; 106.10 ± 9.24 mg glucose/dl). Samples were transferred on dry ice and stored at −80°C until analyzed. The amount of vitreous in the samples varied; consequently, not all samples were analyzed for inflammatory and angiogenic proteins (numbers are indicated in the table and figure legends).

Lipidomics. Frozen vitreous samples (>10 μl) were thawed on ice, and two volumes of methanol and 500 pg each of d_4-PGE2, d_8-12-HETE, d_{11}-11(12)-DiHETrE (dihydroxyeicosatrienoic acid), d_{11}-8(9)-EET, d_6-20-HETE, and d_8-11(12)-EET were added as internal standards. Samples were then centrifuged at 1,500*g* for 15 min at 4°C, and supernatants were diluted with water and acidified to pH 4.0 with 2 mol/l HCl. The pellet was saved and used for protein determination using the Bio-Rad Protein Assay. Solid-phase lipid extraction was performed using C18-ODS AccuBond II 500-mg cartridges (Agilent Technologies, Santa Clara, CA) as described (12). Eicosanoid and docosanoid identification and quantification was carried out using a Q-trap 3200 linear ion trap quadruple liquid chromatography-tandem mass spectrometry (LC/MS/MS) equipped with a Turbo V ion source operated in negative electrospray mode (Applied Biosystems, Foster City, CA) as described (12). Multiple reaction monitoring was used with a dwell time of 25 or 50 ms for each compound with resource parameters: ion spray voltage (4,500 V), curtain gas (40 units), ion source gas flow rate (165 and 250 units), and a temperature of 600°C. Synthetic standards were used to obtain standard curves (5–500 pg) for each compound and internal standard. The given amounts in each sample were corrected for loss during extraction and normalized to protein content.

Qualitative membrane arrays. Preliminary screening was carried out using a large commercial membrane array kit (Human Cytokine Array V; RayBiotech, Norcross, GA) that contained duplicate dots of capture antibodies specific for 79 cytokines, growth factors, and angiogenic modulators on a cadaver vitreous sample as described (13). The results of this analysis identified 12 of the 79 probed proteins as the major components in human vitreous. Therefore, individual and pooled vitreous samples were probed using membrane kits for 43 angiogenic protein modulators (Human Angiogenic array, RayBiotech), which screened for all but two of the 12 detected proteins (human interferon-inducible protein 10 [IP-10] and macrophage inflammatory protein 1α). Analysis was carried out by coupling the sandwich ELISA array kits to a femtogram/ml-sensitive substrate as described (13). Each analytic set was accompanied by a fresh set of two membranes developed in tandem that served as blank controls in the absence of added vitreous. Images were documented on an LAS-4000 mini image station (Fujifilm).

Quantitative micro-well plate-based arrays. Three micro-well arrays were used in this study: *1*) an angiogenic array (Quansys Biosciences, Logan, UT) specific for ANG-2, VEGF, basic fibroblast growth factor (bFGF), platelet-derived growth factor-BB (PDGF-BB), HGF, tissue inhibitors of metalloprotease (TIMP-1 and- 2), tumor necrosis factor (TNF)-α, and VEGF-A; *2*) a chemokine array specific for GROα (growth-regulated oncogene), IL-8, IP-10, MCP-1, RANTES (regulated upon activation, normal T-cell expressed and secreted), and TARC (thymus- and activation-regulated chemokine) (Quansys Biosciences, Logan, UT); and *3*) a custom-designed array specific for VEGFR-1, VEGFR-2, and TNF-R2 (Aushon Biosystems, Boston, MA). The assay protocol and reagents were modified to increase detection sensitivity to the femtogram/ml range as described (14). The plates were imaged using a Fuji film mini Las 4000 imaging station equipped with a high quantum efficiency cooled digital camera with a light panel (LP) plate. Imaging was carried for periods up to 5 min and stored with and without summing. The set of summed images was then examined with specific time points selected for densitometry and quantification based on the degree of saturation for specific proteins of interest. Because the assays for different proteins gave rise to a wide range of densities, several time points were selected for quantitative analysis. The developed arrays were densitometrically analyzed using Array Gauge (Fujifilm) and Q-View Software (Quansys Biosciences).

Statistical analysis. Vitreous samples were analyzed using the Mann-Whitney rank-sum test. A P value <0.05 was considered significant. All data are presented as mean ± SEM.

RESULTS

Vitreous lipid autacoids. The LC/MS/MS-based analysis of lipid autacoids was set to detect COX-, LO-, and CYP-derived arachidonic acid metabolites (eicosanoids) and LO-derived metabolites of docosahexaenoic acid (DHA) including resolvins and neuroprotectin D1 (NPD1) (15) with a sensitivity range of 5–25 pg. COX-derived metabolites including PGE_2/D_2 and TXB_2 were not detected in the vitreous samples. The most recurring eicosanoids in the vitreous were the LO-derived 5-HETE, 12-HETE, 15-HETE, and the CYP-derived EETs including 11(12)-EET, 14(15)-EET, 8(9)-EET, and 5(6)-EET (Fig. 1). DHA metabolites were detected in 8 out of 47 vitreous samples and included 4-HDHA (four samples; 56–3,023 pg/mg), 17-HDHA (two samples; 1,026–9,577 pg/mg), 14-HDHA (five samples; 71–1,220 pg/mg), 7-HDHA (three samples; 28–626 pg/mg), and NPD1 (three samples; 24–91 pg/mg). When combining all DHA-derived resolvins and NPD1, the nondiabetic vitreous ($n = 2$) contained severalfold higher levels than the diabetic vitreous ($n = 6$) (1,826 ± 1,015 and 107 ± 28 pg/mg, respectively).

Among the LO-derived metabolites, 5-HETE (>5 pg) was detectable in 31 out of 47 vitreous samples. More importantly, it exhibited a significant increase in patients with diabetic retinopathy, specifically, in patients with NPDR. 5-HETE was 4.8-fold higher in vitreous from diabetic subjects that from nondiabetic subjects (67.84 ± 11.69 vs. 14.12 ± 6.13 pg/mg protein, mean ± SE, $P = 0.027$) (Fig. 2*A*), and among the four groups, NPDR vitreous contained the highest amount of 5-HETE: 88.56 ± 19.96 pg/mg compared with 4.69 ± 3.69, 26.24 ± 12.15, and 45.73 ± 9.05 pg/mg in ERM, RD, and PDR vitreous, respectively (Fig. 2*B*). Other lipoxygenase-derived eicosanoids including 15- and 12-HETE were detected in fewer samples, 10 (PDR, 5; NPDR, 1; RD, 3; ERM, 1) and 8 (PDR, 7; NPDR, 1; RD, 0; ERM, 0), respectively; the amount

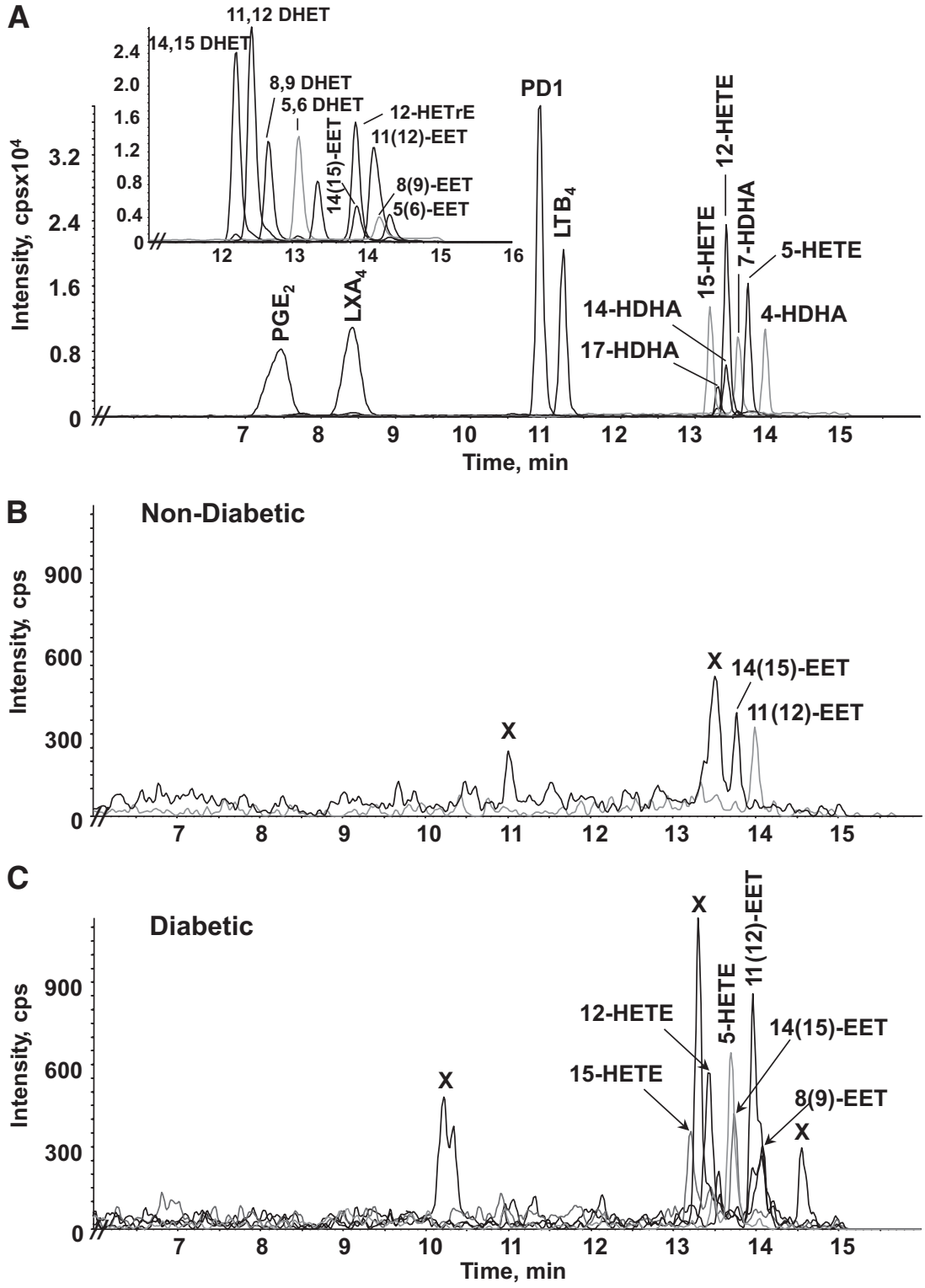


FIG. 1. Representative lipidomics of diabetic and nondiabetic vitreous. Each lipid is identified by its elution time and unique multiple reaction monitoring pair and quantified using standard curve of authentic standards. *A*: Elution profile of authentic standards. *B*: Lipid profile in a nondiabetic vitreous. *C*: Lipid profile of in a diabetic vitreous; x = unknown.

detected ranged from 5 to 350 pg/mg and did not show a distinct pattern.

The vitreous also contained significant levels of CYP-derived EETs and their hydrolytic metabolites dihydroxyeicosatrienoic acids. In fact, their levels were on the average 10 times higher than HETEs and were detected in 20 diabetic and 14 nondiabetic vitreous. The major EETs were 14(15)-EET and 11(12)-EET. Levels of both EETs were substantially higher in the nondiabetic control subjects (Fig. 3*A* and *B*). 14(15)-EET amounted to 1,086 ± 430 and 242 ± 64 pg/mg in the nondiabetic and diabetic vitreous, respectively. Among the nondiabetic groups, RD displayed the highest value of 1,306 ± 630 compared with 758 ± 560 pg/mg in the ERM. Among the diabetic vitreous,

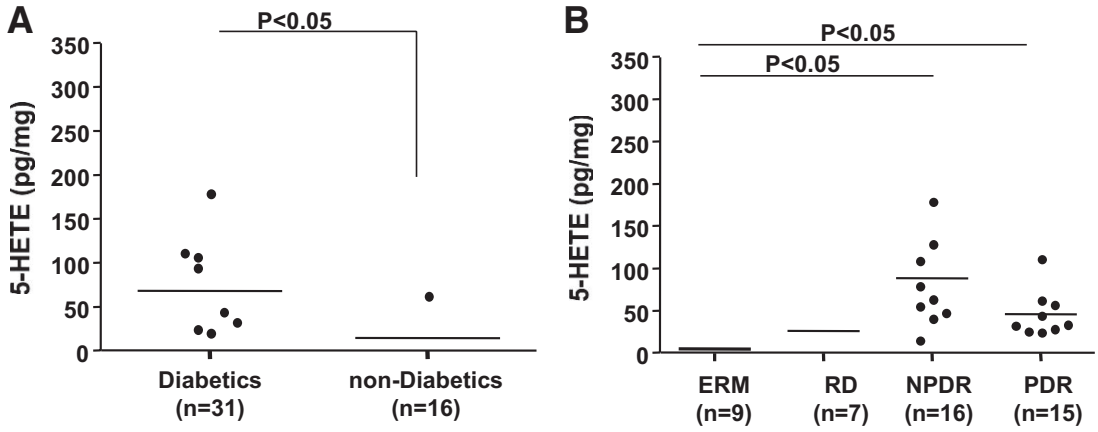


FIG. 2. Levels of 5-HETE in nondiabetic and diabetic vitreous (*A*) and ERM, RD, NPDR, and PDR vitreous (*B*).

the level of 14(15)-EET was the lowest in PDR (147 ± 34 vs. 404 ± 152 pg/mg in NPDR vitreous). 11(12)-EET showed a similar pattern: high levels in nondiabetic (780 ± 287 pg/mg) compared with diabetic (115 ± 27 pg/mg) vitreous, with the PDR displaying the lowest (86 ± 24 pg/mg) and the RD vitreous the highest (1,095 ± 443 pg/mg) (Fig. 3*C* and *D*). 8(9)-EET and 5(6)-EET displayed similar patterns but were detected in fewer samples (not shown).

Vitreous proteins. Our preliminary screening using the Human Cytokine Array of 79 proteins in a vitreous sample obtained from a human cadaver identified 12 proteins: GRO (generic), IL-8, IL-6, IL-1α and -β, MIP-1α, CSF, MCP-1, ANG, leptin, IP-10, and TIMP-1 and -2 in 100 μl vitreous (data not shown). Using a smaller angiogenic array (Fig. 4), detectable signals for GRO (generic), IL-8, IL-6, MCP-1, ANG, leptin, TIMP-1, and TIMP-2 were observed with the relative distribution strikingly different in the pathological and normal (ERM) vitreous samples. The vitreous sample from subjects with ERM exhibited a relatively sparse protein profile consisting of strong signals only for ANG and TIMP1. All of the pathological vitreous samples including RD exhibited to varying degrees strong sets of signals for GRO (generic), IL-8, IL-6, MCP-1, ANG, leptin, TIMP-1, and TIMP-2. We also identified upregulated proteins that included VEGF, leptin, IL-6,

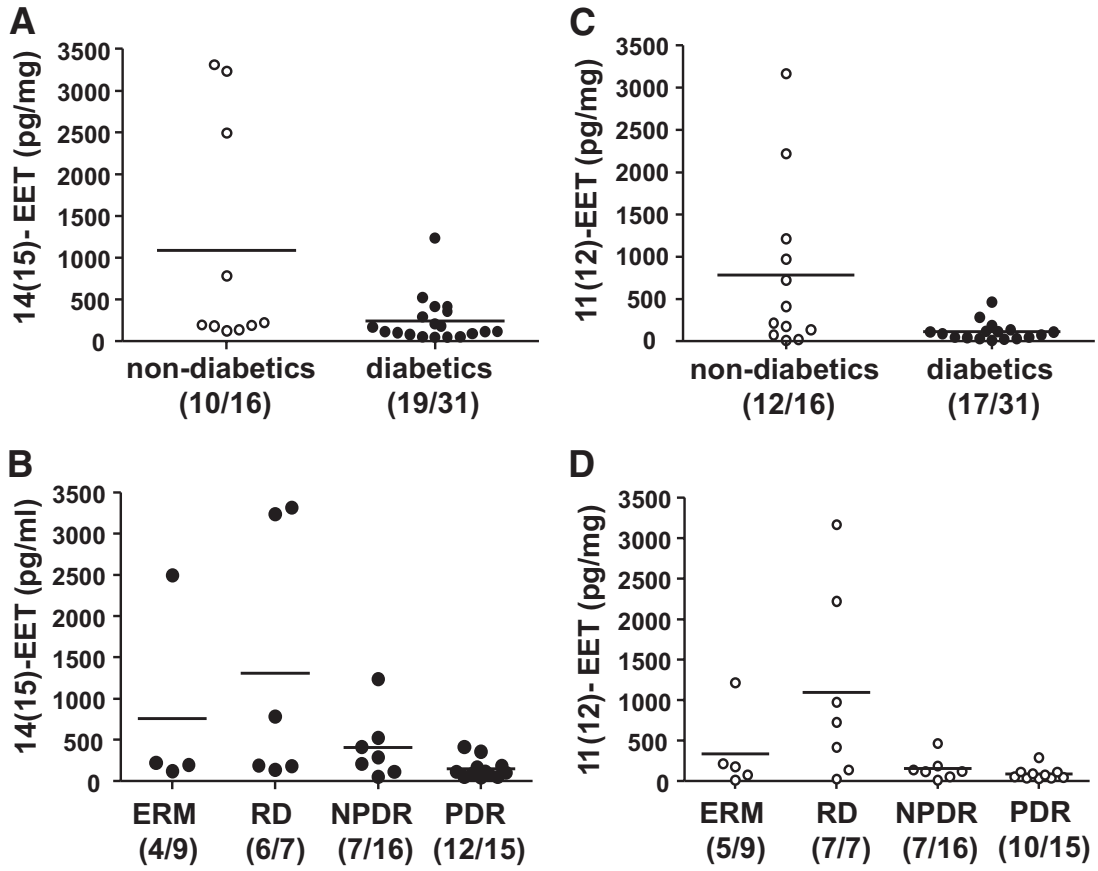
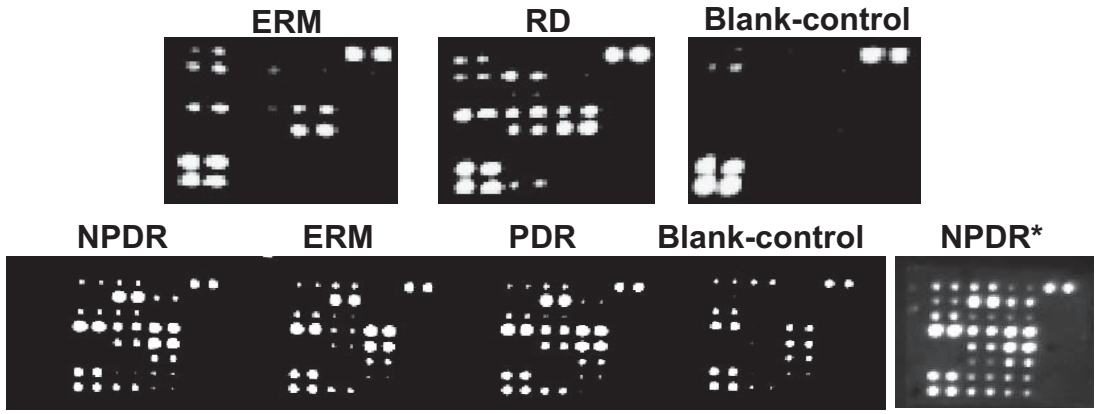


FIG. 3. Levels of 14(15)-EET and 11(12)-EET in nondiabetic and diabetic vitreous and (*A* and *C*, respectively) and in ERM, RD, NPDR, and PDR vitreous (*B* and *D*, respectively).



	1	2	3	4
A	bFGF 1000	PDGF-BB 1000	VEGF-D 1000	Pos
B	ENA-78 1	MCP-1 3	VEGF 100	Neg
C	EGF 1	LEPTIN 100	Thrombopoietin *not known	Blank
D	Angiogenin 10	IL-8 1	Timp-1 100	Blank
E	NEG	IL-6 1	Timp-2 1	Blank
F	NEG	IGF-1 10	TGF-β1 200	Blank
G	POS	IFN-γ 100	RANTES 2000	Blank
H	POS	*GRO	PIG F 100	Blank

FIG. 4. Representative arrays of vitreous samples. *Upper panel*: Representative membranes of vitreous samples (300 μl) from individuals with an ERM and RD sample along with blank control. *Lower panel*: Representative membranes of pooled vitreal samples from individuals randomly selected with NPDR, ERM, and PDR (three samples of 700 μl each in each category) and a membrane probed with 700-μl samples of vitreous from an individual exhibiting a rapid disease progression (NPDR*). Array configuration and the sensitivity limits for each protein determined by the manufacturer are depicted in the table.

IL-8, TIMP-1, and TIMP-2. Particularly striking was the pattern of signals that was unique to the one NPDR subject who experienced a rapid transition to proliferative diabetes (NPDR*). This sample displayed a relatively high signal for VEGF and a very low signal for leptin (Fig. 1).

Quantitative analysis was carried out using micro-well plate array methodology. Analysis of multifunctional chemokines consisting of angiogenic and angiostatic entities revealed measurable levels of GROα, MCP-1, IP-10, IL-8, RANTES, and TARC in most samples with distinct differences in concentration and in the distribution between ERM vitreous and vitreous from RD, NPDR, and PDR (Table 1). GROα was not detected in ERM and PDR vitreous; moreover, levels of MCP-1, IL-8, RANTES, and TARC in ERM were significantly lower than in most of the other groups (Table 1). MCP-1 was present in all vitreous samples, and its concentration in ERM, NPDR, and PDR vitreous was comparable to previously reported values in vitreous of subjects with proliferative vitreoretinopathy and diabetic vitreous (16,17). Interestingly, MCP-1 concentration was significantly higher in RD vitreous displaying five times higher levels than in ERM vitreous and about four times higher than the NPDR and PDR vitreous. Particularly surprising was the marked difference in the distribution of GRO in the NPDR and the PDR populations, suggesting a marked drop off of this chemokine with progression to proliferation.

Additional analysis was carried out using an angiogenic array specific for ANG-2, basic FGF, HGF, PDGF-BB,

TABLE 1
Chemokines concentrations (in pg/ml) in vitreous from subjects with ERM, RD, NPDR, and PDR

	ERM	RD	NPDR	PDR
n	9	6	14	13
GROα	0 ± 0	147 ± 83	104 ± 50	0 ± 0
IL-8	5 ± 3	214 ± 130*	87 ± 42*	52 ± 25*
IP-10	18 ± 7	193 ± 86	48 ± 20	46 ± 19
MCP-1	856 ± 159	4,519 ± 1,136*	1,610 ± 520	1,414 ± 210
RANTES	14 ± 11	70 ± 22	199 ± 176*	21 ± 10*
TARC	4 ± 2	57 ± 17 *	13 ± 7	6 ± 2

Data are expressed in picograms per milliliter and are means ± SE. *n* = number of samples. *$P < 0.05$ vs. ERM.

TABLE 2
Angiogenic and anti-angiogenic factors (in pg/ml) in the vitreous

	ERM	RD	NPDR	PDR
n	8	6	18	12
ANG-2	1,326 ± 650	2,610 ± 740	3,641 ± 1,218	3,528 ± 1,753
Basic FGF	756 ± 756	0 ± 0	636 ± 300	235 ± 235
HGF	27,704 ± 7,912	10,846 ± 1,275*	29,106 ± 3,821	24,786 ± 3,009
PDGF-BB	872 ± 469	0 ± 0	969 ± 360	366 ± 261
TIMP-1	367,841 ± 84,766	567,205 ± 84,921	352,477 ± 50,960	431,310 ± 54,912
TIMP-2	253,031 ± 44,718	126,403 ± 24,625	257,830 ± 21,453	286,248 ± 31,168
TNF-α	31 ± 19	0 ± 0	153 ± 97	125 ± 118
VEGF	0 ± 0	0 ± 0	647 ± 308*	307 ± 159

Data are expressed in picograms per milliliter and are means ± SE. *n* = number of samples. *$P < 0.05$ vs. ERM.

TIMP-1 and -2, TNF-α, and VEGF (Table 2). Significant amounts of these factors were detected in all samples with HGF, TIMP-1, and TIMP-2 present in ng/ml quantities. Among these angiogenic entities, only VEGF showed a significant diabetes-specific profile, although such tendency was also noted for TNF-α. Levels of VEGF in vitreous from subjects with either proliferative or nonproliferative diabetic retinopathy were significantly higher when compared with nondiabetic subjects. Interestingly, VEGF was not detected in vitreous from patients with RD (Table 2).

The angiogenic/inflammatory effect of VEGF and TNF-α largely depends on the concentration of their soluble receptors. Soluble VEGF-R1 and VEGF-R2 as well as TNF-R2 act as traps for their corresponding ligands. A custom-made array was used to determine the levels of these receptors. Soluble VEGF-R1 levels were not significantly different among the groups, whereas levels of soluble VEGF-R2 were significantly lower in NPDR and PDR than in ERM (Table 3). Importantly, the ratio of VEGF to its soluble receptors, VEGF-R1 and VEGF-R2, was higher in PDR and NPDR patients than in control ERM patients (Fig. 5). Soluble TNF-R2 was readily detected in all groups and was significantly higher in PDR when compared with ERM vitreous (Table 3). The ratio of TNF-α to its receptor was higher in NPDR and PDR vitreous but did not reach significance when compared with ERM vitreous.

DISCUSSION

The diabetic vitreous contains elevated levels of many angiogenic and inflammatory proteins and lipids, with some of these changes associated with diabetic progression (6,7). This study compares and contrasts levels of angiogenic and angiostatic proteins and inflammatory and anti-inflammatory lipid mediators, primarily eicosanoids, in the diabetic and nondiabetic vitreous and correlates them with the progression from nonproliferative to proliferative diabetic retinopathy. We clearly demonstrate that changes in these autacoids follow the progression of the disease with a general increase in angiogenic and inflammatory autacoids in the diabetic vitreous.

The present study is the first to perform lipidomic analysis of eicosanoids and docosanoids in vitreous from diabetic and nondiabetic patients and shows that the vitreous contains detectable amounts of these small lipid autacoids. What emerges from this analysis is a profile that is specific for diabetes, indicating a shift in favor of recognized proinflammatory over anti-inflammatory eicosanoids in diabetic vitreous. The cellular source of these lipid autacoids is unknown and could be derived from retinal tissues including the retinal vascular endothelial, glial, and pigmented epithelial cells as well as from infiltrated inflammatory cells. Different patterns of eicosanoids may be seen in these tissues; therefore, what is in the vitreous may not fully reflect the changes at these tissue levels. Nevertheless, the lipid accumulation in the vitreous is still indicative of a distinct paracrine effect on this tissue. The most abundant proinflammatory eicosanoid was the 5-LO-derived 5-HETE. The 5-LO (Alox5) is the initial enzymatic step in the synthesis of leukotrienes (LTs), including LTB_4 and the cysteinyl-LTs, LTC_4, LTD_4, and LTE_4. These 5-LO–derived LTs are strong inflammatory mediators (18). LTB_4 is a potent chemoattractant factor that increases leukocyte aggregation and adhesion to the vascular endothelium. LTC_4 and LTD_4 increase vascular permeability and are potent vasoconstrictors. The role of 5-LO in inflammation is supported by the demonstration that mice null for the 5-LO gene display a reduced inflammatory reaction (19,20). In line with these characteristics, the finding of high levels of 5-HETE in the diabetic vitreous is highly significant. 5-HETE is generally devoid of the powerful inflammatory properties of LTs; however, its presence indicates a considerable 5-LO activity as it is the degradation product of 5-HPETE, the unstable intermediate in the synthesis of LTA_4 and consequently cysteinyl-LTs (21). The vitreous levels of 5-HETE were five times higher in diabetic patients than in the nondiabetic patients. Moreover, within the diabetic group, vitreous from patients with NPDR contained twice as

TABLE 3
VEGF and TNF-α receptor concentrations in the vitreous

	ERM	RD	NPDR	PDR
n	9	6	14	13
TNF-R2	127 ± 12	192 ± 42	199 ± 35	245 ± 32*
VEGF-R1	3,779 ± 360	2,735 ± 333	4,114 ± 830	3,606 ± 607
VEGF-R2	11,823 ± 1,460	8,676 ± 1,239	8,026 ± 1,809*	6,430 ± 728*

Data are expressed in picograms per milliliter and are means ± SE. *n* = number of samples. *$P < 0.05$ vs. ERM.

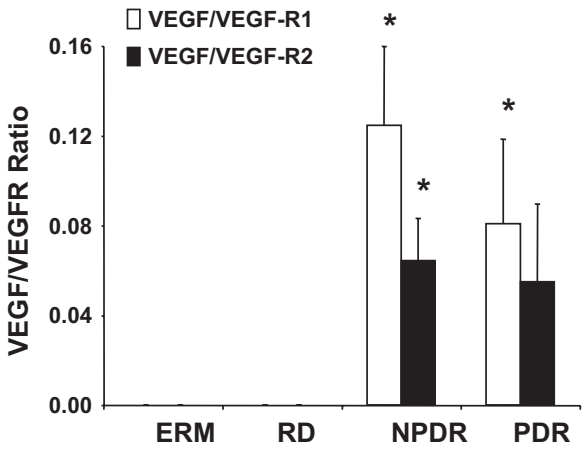


FIG. 5. Ratios of VEGF to VEGFR1 and VEGFR2 in vitreous from patients with ERM, RD, NPDR, and PDR. Results are mean ± SE. *P < 0.05 vs. ERM.

much 5-HETE as the patient with PDR, suggesting that the 5-LO pathway participates in the initiation and amplification of the inflammation. The predominant expression of 5-lipoxygenase in myeloid cells makes infiltrating leukocytes the likely source of 5-HETE. The importance of the leukocyte in the early initiation phase of diabetic retinopathy is an emerging area. Talahalli et al. (22) failed to detect 5-LO in the retina, yet retinal cells could generate LTB_4 and LTC_4 after LTA_4 was provided, suggesting that the likely source of 5-HETE and LTA_4 is circulating leukocytes, and the surrounding retinal cells participate in the amplification of the inflammatory signal (LTB_4) and vascular permeability (LTC_4). The authors concluded that generation of LTs could contribute to chronic inflammation and retinopathy in diabetes. Indeed, Gubitosi-Klug et al. (8) provided convincing evidence for a major role of 5-LO in the development of diabetic retinopathy. Retinas from diabetic 5-LO–deficient mice had significantly less leukostasis, superoxide production, and nuclear factor (NF)-κB expression, all of which are markers for inflammation in early diabetic retinopathy (1). Typical diabetic alterations were also significantly reduced in these mice.

5-LO can also generate the anti-inflammatory metabolites of EPA and DHA, the resolvins and protectins (23), some of which interact with the LTB_4 receptor (BLT1) as receptor antagonists. Their presence and that of lipoxins, 15-LO–derived anti-inflammatory arachidonate metabolites (23), may present a significant counteracting mechanism in the initiation or progression of diabetic retinopathy. 15-HETE and its unstable precursor 15-HPETE, which are known cell growth regulators, have been detected in epiretinal membranes from patients with proliferative vitreoretinopathy and PDR (24). In our study, lipoxin A4 was not detected in any of the vitreous samples, while 15-HETE was present in 10 out of 47 samples with no significant differences among the groups. Likewise, the DHA-derived resolvins and protectins were detected in few samples (six diabetic vitreous and two nondiabetic vitreous). The two nondiabetic vitreous samples contained 18-fold higher levels of these metabolites than the diabetic vitreous. Interestingly, a recent study indicated that the percentage of DHA is significantly decreased in the retina of diabetic mice (25). Certainly, additional studies need to be performed and further linked to dietary intake. It would be interesting to evaluate whether increasing dietary intake of ω-3 fatty acids affects the progression of diabetic retinopathy in view of reports that these lipids reduce pathological angiogenesis such as in retinopathy of prematurity (26) and may act in a protective role against ischemia-, light-, oxygen-, inflammatory-, and age-associated pathology of the vascular and neural retina (27).

The findings that EETs are abundant in the vitreous and that their levels are significantly higher in nondiabetic vitreous than in diabetic vitreous is of great significance. EETs, primarily 11(12)-EET and 14(15)-EET, are potent vasodilators and are endowed with anti-inflammatory, cytoprotective, and neuroprotective properties (28–30). Increasing the levels of EETs by exogenous administration, inhibition of their degradation, or overexpression of their production has anti-inflammatory effects including inhibition of cytokine production, endothelial cell adhesion molecule expression, and leukocyte adhesion to the vascular wall by a mechanism involving NF-κB inhibition (11,31). There are also reports indicating that EETs have neuroprotective properties (32); however, their role in the pathophysiology of the eye and in particular the retina is poorly defined. Interestingly, plasma and tissue concentrations of EETs in a mouse model of type 2 diabetes are significantly lower compared with levels in control mice, and administration of an EET agonist reverses the diabetic states (33). Given the known properties of these metabolites and other lipid autacoids detected in this study, a pattern can be discerned from the lipidomics analysis—the proinflammatory autacoids (in particular, 5-HETE) are far more abundant in diabetic vitreous than in nondiabetic vitreous, while anti-inflammatory autacoids including 15-HETE, resolvins, NPD1, and EETs are notably reduced in diabetic vitreous compared with nondiabetic vitreous.

Analysis of cytokines and chemokines presented a profile similar to that of the lipid autacoids showing a diabetes-dependent increase in key inflammatory and angiogenic factors. Among the vitreous chemokines, only MCP-1, IP-10, IL-8, and GROα were substantially expressed, while levels of RANTES and TARC were occasionally detected. MCP-1 was detected in all samples as reported by others (16,17). ERM samples had chemokine levels that were a small fraction of those in the other groups. The ERM patients had a total of 1,005 pg/ml in their combined samples or 126 pg/ml per patient. NPDR patients had 339 pg/ml per patient, and PDR patients had 197 pg/ml per patient. Thus, there appears to be an increase in overall chemokine levels in diabetic retinopathy. RD patients had, by far, the highest chemokine levels: 678 pg/ml per patient, including significant 5- to 40-fold higher levels of MCP-1, TARC, and IL-8 when compared with ERM vitreous. RD is characterized by the apoptotic death of the outer retinal layers; neovascularization is not a feature of this condition. MCP-1 expression has been reported to be critical for RD-induced photoreceptor apoptosis and subsequent macrophage/microglia infiltration and activation (34). IP-10 is a potent angiostatic factor, and GROα was reported to be angiostatic in high concentrations (35). Thus, angiostatic chemokines dominate in the RD vitreous. The finding in RD vitreous of elevated levels of TARC, a member of the C-C chemokine family and a potent chemoattractant for Th2 cells, is interesting; however, its significance is unclear.

Assessment of distinct angiogenic factors showed that VEGF was the only angiogenic factor that displayed a diabetes-specific distribution, although TNF-α showed the same profile but did not achieve significance. Levels of

VEGF in ERM and RD vitreous were below detection levels, while it was readily detected in vitreous from NPDR and PDR subjects (4,17,36,37). Likewise, TNF-α, which is present at a high concentration in PDR vitreous (38,39), was only detected in vitreous from NPDR and PDR subjects. Interestingly, soluble receptors for VEGF and TNF-α were prominent in the vitreous from all groups. These soluble receptors are considered endogenous inhibitors or traps for their corresponding ligands, and some have been detected in vitreous from patients with proliferative vitreoretinopathy (36,40). Levels of soluble VEGF-R2 were significantly lower in NPDR and PDR vitreous when compared with ERM vitreous, suggesting a lower capacity to quench the bioactivity of VEGF in the diabetic retina. On the other hand, levels of VEGF-R1 were similar in diabetic and nondiabetic subjects. Matsunaga et al. (36) examined the levels of soluble VEGF-R1 in vitreous from patients with PDR or idiopathic macular hole and found twofold higher levels in PDR. In the present study, while the levels of soluble VEGFR1 were not different, the VEGF/VEGF-R1 ratio was severalfold higher in NPDR and PDR subjects, as was the VEGF/VEGF-R2. The relative lower concentration of soluble VEGF receptors may drive the angiogenic phenotype of the retina in diabetic retinopathy. It should be noted that, in this study, we measured VEGF-A; however, other forms of VEGF may be present in the vitreous of diabetic patients, the levels of which may contribute significantly to the pathology seen in the diabetic eye.

Our study adds support to the role of inflammation in the genesis of diabetic retinopathy. Understanding the implication of these potent lipid and protein autacoids in this condition through studies in transgenic animal models and human studies that take into consideration diets and genetic polymorphisms may provide diagnostic tools and therapeutic targets for treatment and prevention of diabetic retinopathy.

ACKNOWLEDGMENTS

This study was supported by National Institutes of Health Grants EY06513 and HL34300.

No potential conflicts of interest relevant to this article were reported.

M.L.S. researched data and wrote the manuscript; P.I., K.G., L.B., S.S., A.B., and L.T. researched data and reviewed/edited the manuscript; A.L. coordinated the sample collection, researched data, and reviewed/edited the manuscript; M.S. and M.G. collected samples, researched data, and reviewed/edited the manuscript; M.W.D. contributed to the discussion and reviewed/edited the manuscript; and R.S. researched data and reviewed/edited the manuscript.

We wish to express our gratitude to Dr. Monica Berry for helpful suggestions in reviewing an earlier draft of this manuscript.

This study was presented in abstract form at the 2009 meeting of the Association for Research in Vision and Ophthalmology.

REFERENCES

1. Kern TS. Contributions of inflammatory processes to the development of the early stages of diabetic retinopathy. Exp Diabetes Res 2007;2007:95103
2. Joussen AM, Poulaki V, Le ML, Koizumi K, Esser C, Janicki H, Schraermeyer U, Kociok N, Fauser S, Kirchhof B, Kern TS, Adamis AP. A central role for inflammation in the pathogenesis of diabetic retinopathy. Faseb J 2004;18:1450–1452
3. Banerjee S, Savant V, Scott RA, Curnow SJ, Wallace GR, Murray PI. Multiplex bead analysis of vitreous humor of patients with vitreoretinal disorders. Invest Ophthalmol Vis Sci 2007;48:2203–2207
4. Murugeswari P, Shukla D, Rajendran A, Kim R, Namperumalsamy P, Muthukkaruppan V. Proinflammatory cytokines and angiogenic and anti-angiogenic factors in vitreous of patients with proliferative diabetic retinopathy and eales' disease. Retina 2008;28:817–824
5. Spranger J, Osterhoff M, Reimann M, Möhlig M, Ristow M, Francis MK, Cristofalo V, Hammes HP, Smith G, Boulton M, Pfeiffer AF. Loss of the antiangiogenic pigment epithelium-derived factor in patients with angiogenic eye disease. Diabetes 2001;50:2641–2645
6. Simó R, Carrasco E, García-Ramírez M, Hernández C. Angiogenic and antiangiogenic factors in proliferative diabetic retinopathy. Curr Diabetes Rev 2006;2:71–98
7. Hardy P, Beauchamp M, Sennlaub F Jr, Gobeil F, Mwaikambo B, Lachapelle P, Chemtob S. Inflammatory lipid mediators in ischemic retinopathy. Pharmacol Rep 2005;57(Suppl.):169–190
8. Gubitosi-Klug RA, Talahalli R, Du Y, Nadler JL, Kern TS. 5-Lipoxygenase, but not 12/15-lipoxygenase, contributes to degeneration of retinal capillaries in a mouse model of diabetic retinopathy. Diabetes 2008;57:1387–1393
9. Laniado Schwartzman M, Green K, Edelhauser HF, Hackett RB, Hull DS, Potter DE, Tripathi RC. In *Cytochrome P450 and Arachidonic Acid Metabolism in the Corneal Epithelium: Role in Inflammation.* New York, Plenum Press, 1997, p. 3–20
10. Gronert K. Lipoxins in the eye and their role in wound healing. Prostaglandins Leukot Essent Fatty Acids 2005;73:221–229
11. Node K, Huo Y, Ruan X, Yang B, Spiecker M, Ley K, Zeldin DC, Liao JK. Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. Science 1999;285:1276–1279
12. Patil K, Bellner L, Cullaro G, Gotlinger KH, Dunn MW, Schwartzman ML. Heme oxygenase-1 induction attenuates corneal inflammation and accelerates wound healing after epithelial injury. Invest Ophthalmol Vis Sci 2008;49:3379–3386
13. Sack RA, Conradi L, Krumholz D, Beaton A, Sathe S, Morris C. Membrane array characterization of 80 chemokines, cytokines, and growth factors in open- and closed-eye tears: angiogenin and other defense system constituents. Invest Ophthalmol Vis Sci 2005;46:1228–1238
14. Li S, Sack R, Vijmasi T, Sathe S, Beaton A, Quigley D, Gallup M, McNamara NA. Antibody protein array analysis of the tear film cytokines. Optom Vis Sci 2008;85:653–660
15. Mukherjee PK, Marcheselli VL, Serhan CN, Bazan NG. Neuroprotectin D1: a docosahexaenoic acid-derived docosatriene protects human retinal pigment epithelial cells from oxidative stress. Proc Natl Acad Sci U S A 2004;101:8491–8496
16. Capeans C, De Rojas MV, Lojo S, Salorio MS. C-C chemokines in the vitreous of patients with proliferative vitreoretinopathy and proliferative diabetic retinopathy. Retina 1998;18:546–550
17. Maier R, Weger M, Haller-Schober EM, El-Shabrawi Y, Wedrich A, Theisl A, Aigner R, Barth A, Haas A. Multiplex bead analysis of vitreous and serum concentrations of inflammatory and proangiogenic factors in diabetic patients. Mol Vis 2008;14:637–643
18. Lewis RA, Austen KF, Soberman RJ. Leukotrienes and other products of the 5-lipoxygenase pathway: biochemistry and relation to pathobiology in human diseases. N Engl J Med 1990;323:645–655
19. Chen XS, Sheller JR, Johnson EN, Funk CD. Role of leukotrienes revealed by targeted disruption of the 5-lipoxygenase gene. Nature 1994;372:179–182
20. Funk CD, Chen XS. 5-Lipoxygenase and leukotrienes: transgenic mouse and nuclear targeting studies. Am J Respir Crit Care Med 2000;161:S120–S124
21. Rubin P, Mollison KW. Pharmacotherapy of diseases mediated by 5-lipoxygenase pathway eicosanoids. Prostaglandins Other Lipid Mediat 2007;83:188–197
22. Talahalli R, Zarini S, Sheibani N, Murphy RC, Gubitosi-Klug RA. Increased synthesis of leukotrienes in the mouse model of diabetic retinopathy. Invest Ophthalmol Vis Sci 51:1699–1708
23. Serhan CN, Chiang N, Van Dyke TE. Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators. Nat Rev Immunol 2008;8:349–361
24. Augustin AJ, Grus FH, Koch F, Spitznas M. Detection of eicosanoids in epiretinal membranes of patients suffering from proliferative vitreoretinal diseases. Br J Ophthalmol 1997;81:58–60
25. Hegde KR, Varma SD. Electron impact mass spectroscopic studies on mouse retinal fatty acids: effect of diabetes. Ophthalmic Res 2009;42:9–14
26. Connor KM, SanGiovanni JP, Lofqvist C, Aderman CM, Chen J, Higuchi A, Hong S, Pravda EA, Majchrzak S, Carper D, Hellstrom A, Kang JX, Chew EY, Salem N Jr, Serhan CN, Smith LE. Increased dietary intake of

omega-3-polyunsaturated fatty acids reduces pathological retinal angiogenesis. Nat Med 2007;13:868–873
27. SanGiovanni JP, Chew EY. The role of omega-3 long-chain polyunsaturated fatty acids in health and disease of the retina. Prog Retin Eye Res 2005;24:87–138
28. Spector AA. Arachidonic acid cytochrome P450 epoxygenase pathway. J Lipid Res 2009;50(Suppl.):S52–S56
29. Spiecker M, Liao JK. Vascular protective effects of cytochrome p450 epoxygenase-derived eicosanoids. Arch Biochem Biophys 2005;433:413–420
30. Deng Y, Theken KN, Lee CR. Cytochrome P450 epoxygenases, soluble epoxide hydrolase, and the regulation of cardiovascular inflammation. J Mol Cell Cardiol 2010;48:331–341
31. Moshal KS, Zeldin DC, Sithu SD, Sen U, Tyagi N, Kumar M, Hughes WM, Jr, Metreveli N, Rosenberger DS, Singh M, Vacek TP, Rodriguez WE, Ayotunde A, Tyagi SC. Cytochrome P450 (CYP) 2J2 gene transfection attenuates MMP-9 via inhibition of NF-kappabeta in hyperhomocysteinemia. J Cell Physiol 2008;215:771–781
32. Zhang W, Otsuka T, Sugo N, Ardeshiri A, Alhadid YK, Iliff JJ, DeBarber AE, Koop DR, Alkayed NJ. Soluble epoxide hydrolase gene deletion is protective against experimental cerebral ischemia. Stroke 2008;39:2073–2078
33. Sodhi K, Inoue K, Gotlinger K, Canestraro M, Vanella L, Kim DH, Manthati VL, Koduru SR, Falck JR, Schwartzman ML, Abraham NG. Epoxyeicosatrienoic acid agonist rescues the metabolic syndrome phenotype of HO-2-null mice. J Pharmacol Exp Ther 2009;331:906–916
34. Nakazawa T, Hisatomi T, Nakazawa C, Noda K, Maruyama K, She H, Matsubara A, Miyahara S, Nakao S, Yin Y, Benowitz L, Hafezi-Moghadam A, Miller JW. Monocyte chemoattractant protein 1 mediates retinal detachment-induced photoreceptor apoptosis. Proc Natl Acad Sci U S A 2007;104:2425–2430
35. Cao Y, Chen C, Weatherbee JA, Tsang M, Folkman J. Gro-beta, a -C-X-C-chemokine, is an angiogenesis inhibitor that suppresses the growth of Lewis lung carcinoma in mice. J Exp Med 1995;182:2069–2077
36. Matsunaga N, Chikaraishi Y, Izuta H, Ogata N, Shimazawa M, Matsumura M, Hara H. Role of soluble vascular endothelial growth factor receptor-1 in the vitreous in proliferative diabetic retinopathy. Ophthalmology 2008;115:1916–1922
37. Patel JI, Tombran-Tink J, Hykin PG, Gregor ZJ, Cree IA. Vitreous and aqueous concentrations of proangiogenic, antiangiogenic factors and other cytokines in diabetic retinopathy patients with macular edema: implications for structural differences in macular profiles. Exp Eye Res 2006;82:798–806
38. Adamiec-Mroczek J, Oficjalska-Młyńczak J. Assessment of selected adhesion molecule and proinflammatory cytokine levels in the vitreous body of patients with type 2 diabetes: role of the inflammatory-immune process in the pathogenesis of proliferative diabetic retinopathy. Graefes Arch Clin Exp Ophthalmol 2008;246:1665–1670
39. Demircan N, Safran BG, Soylu M, Ozcan AA, Sizmaz S. Determination of vitreous interleukin-1 (IL-1) and tumour necrosis factor (TNF) levels in proliferative diabetic retinopathy. Eye 2006;20:1366–1369
40. Limb GA, Hollifield RD, Webster L, Charteris DG, Chignell AH. Soluble TNF receptors in vitreoretinal proliferative disease. Invest Ophthalmol Vis Sci 2001;42:1586–1591