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

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Running Title: Vitreal lipid and protein autacoids in diabetic retinopathy

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**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 non-proliferative diabetic retinopathy (NPDR), 15 had proliferative diabetic retinopathy (PDR), 7 had retinal detachments (RD) and 9 had epiretinal membranes (ERM). 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-HETE, which demonstrated a diabetes-specific increase (p=0.027) with the highest increase in NPDR vitreous. Vitreous also contained CYP-derived EETs; their levels were higher in non-diabetic as compared to diabetic vitreous (p<0.05). Among inflammatory, angiogenic and angiostatic cytokines and chemokines, only VEGF showed a significant diabetic-specific profile (p<0.05), although a similar trend was noted for TNFα. 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 diabetic-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.
The evolving concept that there is an inflammatory basis for diabetic retinopathy in its early stages characterized by over-expression of pro-inflammatory factors has gained much support and has led to the identification of potent pro-inflammatory transcription factors, chemokines and cytokines in diabetic retinas and vitreous (1-3). There are two main stages of diabetic retinopathy—the earliest, non-proliferative 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 non-inclusive list of up-regulated 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 monoxygenase (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 pro-inflammatory and angiogenic eicosanoids include the COX-derived prostaglandins (PGE2 and TxB2), the LO-derived leukotrienes, and the CYP-derived 12(R)-hydroxyeicosatrienoic acid (12-HETE) (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 non-diabetic patients with retinal detachment (RD) or from patients undergoing epiretinal membrane surgery (ERM) and from diabetic patients with PDR and NPDR were analyzed. This study is the first to demonstrate diabetic-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

Patients. 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 year old) were diabetic with fasting blood glucose at the time of surgery averaging 169.40±12.71 mg/dL (p<0.02 vs non-diabetics), 16 (68±2 year old; 166.50±24.89 mg glucose/dL) had non-proliferative diabetic retinopathy (NPDR) and 15 (63±3 year old; 171.50±13.20 mg glucose/dL) had proliferative diabetic retinopathy (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 non-insulin-dependent diabetes mellitus (NIDDM, n=28) or insulin-dependent diabetes mellitus (IDDM, n=3). The remaining 16 non-diabetic subjects (64±4 year old) with blood glucose of 120.50±12.26 mg/dL underwent vitreo-retinal surgery for repair of a retinal detachment (RD, n=7; 55±7 year old; 142.00±26.33 mg glucose/dL) or surgery for an epiretinal membrane (ERM, n=9; 71±2 year 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 tables and figure legends).

Lipidomics. Frozen vitreous samples (>10 µl) were thawed on ice, two volumes of methanol and 500 pg each of d4-PGE2, d8-12-HETE, d11-11(12)-DiHETErE, d11-8(9)-EET, d6-20-HETE and d8-11(12)-EET were added as internal standards. Samples were then centrifuged at 1500xg for 15 minutes at 4°C and supernatants were diluted with water and acidified to pH 4.0 with 2M HCl. The pellet was saved and used for protein determination using the Biorad 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 quadrupole 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 (MRM) was used with a dwell time of 25 or 50 msec for each compound with resource parameters: ion spray voltage-4500V; curtain gas 40U; ion source gas flow rate 165 and 250 U, and 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 Inc., 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 a 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: angiopoietin-2 (ANG-2), VEGF, basic fibroblast growth factor (bFGF), platelet-derived growth factor-BB (PDGF-BB), HGF, tissue inhibitors of metalloprotease (TIMP-1 and 2), TNFα and VEGF-A; 2) a chemokine array specific for GROα, IL-8, IP-10, MCP-1, RANTES and TARC (thymus- and activation-regulated chemokine); (Quansys Biosciences, Logan, UT); and 3) a custom-designed array specific for VEGFR-1, VEGFR-2, 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 LP plate. Imaging was carried for periods up to 5 minutes and stored with and without summing. The set of summed images was then examined with specific time points selected for densitometry and quantification based upon the degree of saturation for specific proteins of interest. Since 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 less than 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 PGE2/D2 and TXB2 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 epoxyeicosatrienoic acids (EETs) including 11(12)-EET, 14(15)-EET, 8(9)-EET and 5(6)-EET (Figure 1). DHA metabolites were detected in 8 out of 47 vitreous samples and included 4-HDHA (4 samples; 56-3023 pg/mg), 17-HDHA (2 samples; 1026-9577 pg/mg), 14-HDHA (5 samples; 71-1220 pg/mg), 7-HDHA (3 samples; 28-626 pg/mg) and NPD1 (3 samples; 24-91 pg/mg). When combining all DHA-derived resolvins and NPD1 the non-diabetic vitreous (n=2) contained several fold higher levels compared to the diabetic vitreous (n=6) (1826±1015 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 compared to non-diabetics (67.84±11.69 vs. 14.12±6.13 pg/mg protein, mean±SE, p=0027) (Figure 2A) and among the four groups, NPDR vitreous contained the highest amount of 5-HETE, 88.56±19.96 pg/mg compared to 4.69±3.69, 26.24±12.15 and 45.73±9.05 pg/mg in ERM, RD and PDR.
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Vitreous respectively (Figure 2B). 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 detected ranged from 5-350 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 (diHETrEs). In fact, their levels were on the average 10 times higher than HETEs and were detected in 20 diabetic and 14 non-diabetic vitreous. The major EETs were 14(15)-EET and 11(12)-EET. Levels of both EETs were substantially higher in the non-diabetic controls (Figure 3A and B). 14(15)-EET amounted to 1086±430 and 242±64 pg/mg in the non-diabetic and diabetic vitreous, respectively. Among the non-diabetic groups, RD displayed the highest value of 1306±630 compared to 758±560 pg/mg in the ERM. Among the diabetic vitreous, the level of 14(15)-EET was the lowest in PDR, 147±34 pg/mg vs. 404±152 pg/mg in NPDR vitreous. 11(12)-EET showed a similar pattern; high levels in non-diabetic (780±287 pg/mg) compared to diabetic (115±27 pg/mg) vitreous with the PDR displaying the lowest (86±24 pg/mg) and the RD vitreous the highest (1095±443 pg/mg) (Figure 3C 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, TIMP-1 and 2 in 100 µl of vitreous (data not shown). Using a smaller angiogenic array (Figure 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 up-regulated proteins that included VEGF, leptin, IL-6, IL-8, TIMP-1 and 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 (Figure 1).

Quantitative analysis was carried out using microwell 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 5-times higher levels than in ERM vitreous and about 4-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, TIMP-1 and 2, TNFα 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 diabetic-specific profile, although such tendency was also noted for TNFα. Levels of VEGF in vitreous from subjects with either proliferative or non-proliferative diabetic retinopathy were significantly higher when compared to non-diabetic subjects. Interestingly, VEGF was not detected in vitreous from patients with RD (Table 2).

The angiogenic/inflammatory effect of VEGF and TNFα is largely dependent 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 compared to ERM (Table 3). Importantly, the ratio of VEGF to its soluble receptors, VEGF-R1 and VEGF-R2, was higher in PDR and NPDR patients compared to control ERM patients (Figure 5). Soluble TNF-R2 was readily detected in all groups and was significantly higher in PDR when compared to ERM vitreous (Table 3). The ratio of TNFα to its receptor was higher in NPDR and PDR vitreous but did not reach significance when compared to 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 non-diabetic vitreous and correlates them with the progression from non-proliferative 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 non-diabetic 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 pro-inflammatory 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 pro-inflammatory eicosanoid was the 5-LO-derived 5-HETE. The 5-LO (Alox5) is the initial enzymatic step in the synthesis of leukotrienes (LTs) including LTB4 and the cysteinyl-LTs, LTC4, LTD4 and LTE4. These 5-LO-derived LTs are strong inflammatory mediators (18). LTB4 is a potent chemoattractant factor, which increases leukocyte aggregation and adhesion to the vascular endothelium. LTC4 and LTD4 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 LTA₄ and consequently cysteiny-LTs (21). The vitreous levels of 5-HETE were 5 times higher in diabetic patients compared to the non-diabetic patients. Moreover, within the diabetic group, vitreous from patients with NPDR contained twice as much 5-HETE as the patient with PDR, suggesting that 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 LTB₄ and LTC₄ after LTA₄ was provided suggesting that the likely source of 5-HETE and LTA₄ is circulating leukocytes, and the surrounding retinal cells participate in the amplification of the inflammatory signal (LTB₄) and vascular permeability (LTC₄). 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-κB (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 LTB₄ 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 were the DHA-derived resolvins and protectins; they were detected in few samples (6 diabetic vitreous and 2 non-diabetic vitreous). The two non-diabetic 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 non-diabetic 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 to 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 pro-inflammatory autacoids in particular 5-HETE are far more abundant in diabetic vitreous than in non-diabetic vitreous while anti-inflammatory autacoids including 15-HETE, resolvins, NPD1 and EETs are notably reduced in diabetic vitreous compared to non-diabetic 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 1005 pg/ml in their combined samples or 126 pg/ml/patient. NPDR patients had 339 pg/ml/patient and PDR patients had 197 pg/ml/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/patient including significant 5-40-fold higher levels of MCP-1, TARC and IL-8 when compared to 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 (Thymus- and activation-regulated chemokine), 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 TNFα 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, TNFα, which is present at high concentration in PDR vitreous (38; 39), was only detected in vitreous from NPDR and PDR subjects. Interestingly, soluble receptors for VEGF and TNFα 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 to ERM vitreous suggesting a lower capacity to
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quench the bioactivity of VEGF in the diabetic retina. On the other hand, levels of VEGF-R1 were similar in diabetic and non-diabetic subjects. Matsunaga et al (36) examined the levels of soluble VEGF-R1 in vitreous from patients with PDR or idiopathic macular hole and found 2-fold higher levels in PDR. In the present study, while the levels of soluble VEGF-R1 were not different, the VEGF/VEGF-R1 ratio was several-fold 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.

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**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 | 4519 ± 1136 * | 1610 ± 520 | 1414 ± 210 |
| RANTES | 14 ± 11 | 70 ± 22 | 199 ± 176 * | 21 ± 10 * |
| TARC  | 4 ± 2 | 57 ± 17 * | 13 ± 7 | 6 ± 2 |

Results are expressed in pg/ml and are the mean±SE; n=number of samples; *p<0.05 vs. ERM

**Table 2.** Angiogenic and anti-angiogenic factors (pg/ml) in the vitreous

|       | ERM   | RD    | NPDR  | PDR  |
|-------|-------|-------|-------|------|
| n     | (8)   | (6)   | (18)  | (12) |
| ANG-2 | 1326 ± 650 | 2610 ± 740 | 3641 ± 1218 | 3528 ± 1753 |
| Basic FGF | 756 ± 756 | 0 ± 0 | 636 ± 300 | 235 ± 235 |
| HGF   | 27704 ± 7912 | 10846 ± 1275 * | 29106 ± 3821 | 24786 ± 3009 |
| PDGF-BB | 872 ± 469 | 0 ± 0 | 969 ± 360 | 366 ± 261 |
| TIMP-1 | 367841 ± 84766 | 567205 ± 84921 | 352477 ± 50960 | 431310 ± 54912 |
| TIMP-2 | 253031 ± 44718 | 126403 ± 24625 | 257830 ± 21453 | 286248 ± 31168 |
| TNFα  | 31 ± 19 | 0 ± 0 | 153 ± 97 | 125 ± 118 |
| VEGF  | 0 ± 0 | 0 ± 0 | 647 ± 308 * | 307 ± 159 |

Results are expressed in pg/ml and are the mean±SE; n=number of samples; *p<0.05 vs. ERM

**Table 3:** VEGF and TNFα 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 | 3779 ± 360 | 2735 ± 333 | 4114 ± 830 | 3606 ± 607 |
| VEGF-R2 | 11823 ± 1460 | 8676 ± 1239 | 8026 ± 1809 * | 6430 ± 728 * |

Results are expressed in pg/ml and are the mean±SE; n=number of samples; * p<0.05 vs. ERM.
FIGURE LEGENDS

**Figure 1:** Representative lipidomics of diabetic and non-diabetic vitreous. Each lipid is identified by its elution time and unique MRM pair and quantified using standard curve of authentic standards. A) Elution profile of authentic standards; B) Lipid profile in a non-diabetic vitreous; C) Lipid profile of in a diabetic vitreous; x=unknown.

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

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

**Figure 4:** Representative arrays of vitreous samples. Upper panel, representative membranes of vitreous samples (300 µl) from individuals with 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 (3 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.

**Figure 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.
Figure 1

A

B

Non-Diabetic

C

Diabetic

Vitreal lipid and protein autacoids in diabetic retinopathy
Figure 2

![Figure 2](image1)

Figure 3

![Figure 3](image2)
Figure 4

![Image of experimental results](image)

|   | 1       | 2       | 3       | 4       |
|---|---------|---------|---------|---------|
| A | bFGF 1000 | PDGF-EB 1000 | VEGF-D 1000 | Pos     |
| B | ENA-78 1 | MCP-1 3 | VEGF 100 | Neg     |
| C | EGF 1 | LEPTIN 100 | Thrombopoietin \(\text{**not known**}\) | Blank   |
| D | Angiogenin 10 | IL-8 1 | Timp-1 100 | Blank   |
| E | NEG 1 | IL-6 1 | Timp-2 1 | Blank   |
| F | NEG 1 | IGF-1 10 | TGF-\(\beta\)1 200 | Blank   |
| G | PCS 100 | IFN-\(\gamma\) 100 | RANTES 2000 | Blank   |
| H | PCS | *GRO 100 | VEGF 100 | Blank   |

Figure 5

![Image of VEGF/VEGF-R ratio](image)