Intraocular concentrations of cytokines, chemokines, and growth factors in the different forms of retinal detachment and the effect of the macular position

Ph.D. Thesis

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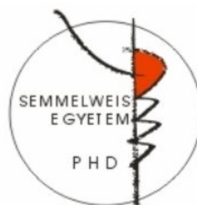

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Introduction

Retinal detachment (RD) is the separation of the neurosensory retina from the underlying retinal pigment epithelium (RPE). RD can cause vision loss if untreated, and even with proper surgical intervention, a potentially sight-threatening condition may develop in some cases.

The most difficult challenges for vitreoretinal surgeons are proliferative vitreoretinopathy (PVR) developed from rhegmatogenous RD (RRD) and proliferative diabetic retinopathy (PDR) complicated with tractional RD. PDR is characterized by neovascularization on the retina and the formation of fibrovascular membranes at the vitreoretinal interface. Complex pathophysiological mechanisms triggered by hyperglycaemia underlie the development of PDR. These mechanisms include hypoxia, the release of inflammatory factors, and vascular endothelial growth factor (VEGF). The development of fibrovascular tissue often leads to hemorrhage and tractional RD. (Wong, Cheung et al. 2016)

In RRD, liquified vitreous enters under the neurosensory retina through a retinal break. When the vitreous reaches the retinal cells, the affected cells start to secrete factors involved in the destruction and survival of retinal structures. (Pollreisz, Sacu et al. 2015)

Kaufman et al. were among the first to report that macular involvement and duration of RRD were major parameters for postoperative visual acuity. (Kaufman 1976) Despite anatomically successful RD surgery

resulting in reattached retina visual acuity remains impaired in almost 40% of cases, especially when the macula was detached or PVR developed after surgery. (Pastor, Fernandez et al. 2008) PVR is based on the development of fibrocellular membranes on the surface of and under the retina after RRD, and it occurs in an estimated 5-10 %. (Pastor 1998; Chaudhary et al. 2020) Various preoperative and postoperative risk factors for the development of PVR are known. Preoperative risk factors include the existence of large retinal tears, a longstanding retinal detachment, vitreous hemorrhage, aphakia, and choroidal detachment. The intraoperative risk factors that mainly influence the development of PVR include the preoperative existence of PVR, inflammation, vitreous hemorrhage, excessive photocoagulation or cryotherapy, incomplete vitrectomy, undetected breaks. (Garweg, Tappeiner et al. 2013) The term PVR was created in 1983 by the Retina Society Terminology Committee which revised the classification Machemer proposed in 1978. In 1991 an updated classification of RD with PVR was also made by Machemer, which is present in Practical Atlas of Retinal Disease and Therapy. (Glazer, Abrams 1993) Machemer was the inventor of the vitreous infusion suction cutter which surgical device made possible the first pars plana vitrectomy (PPV) on 20 April 1970. (Machemer, Buettner et al. 1971) This surgical approach revolutionized the treatment of RRD and other posterior segment diseases. Moreover, Machemer among others studied the pathophysiology of PVR in animal models.

Because of the difficulties in the treatment of PVR and PDR, the pathophysiology of these diseases is under extensive research including cytokines, chemokines, and other inflammatory factors. Many studies have reported an immunological component responsible for PVR, and the formation of tractional RD in PDR. In the first studies, only a few proteins could be assayed in one sample by enzyme-linked immunosorbent assay (ELISA). (Kauffmann, van Meurs et al. 1994, Elner, Elner et al. 1995, Abu El-Asrar, Struyf et al. 2006) Caepaens et al. were among the first to evaluate three chemokines with ELISA in vitreous samples and found that the MCP-1 level was significantly higher in PVR and PDR compared to controls. (Capeans, De Rojas et al. 1998)

Nowadays a new technique, multiplex bead-based immunoassay provides an opportunity to perform a wide range of molecular analyses in one sample. This helps us understand the interaction between the components of the immunological processes responsible for pathological changes in PDR and PVR. (Ricker, Kessels et al. 2012, Dai, Wu et al. 2015) Clinical evidence comparing intraocular cytokine, chemokine, and growth factor levels in patients with PVR, PDR, and RRD is scarce. The role of immunological factors in the pathophysiology of different RDs is important to know to be able to invent new therapeutic targets.

Objectives

Our purposes were:

1. Investigation of the intraocular concentrations of cytokines, chemokines, and growth factors in RRD, PVR, and PDR.
   1.1. Exploration of the immunological components of the vitreous that are responsible for the proliferative alterations in PVR and PDR.
   1.2. Gaining more detailed information and compare the differences in the levels of cytokines, chemokines, and growth factors in the vitreous among the different forms of RD.
2. Subgroup analysis and comparison of the intraocular concentrations of cytokines in eyes with PVR, macula on, and macula off RRD.
   2.1. Defining the intravitreal cytokine, chemokine, growth factor patterns of RRD and PVR.
   2.2. Finding correlation of intravitreal cytokine expression with the position of macula lutea and presence of PVR.

Hypotheses:

1. Patients with macula off RRD and PVR have higher levels of cytokines compared to patients with macula on RRD.

2. There is a correlation between intravitreal cytokine expression and the position of the macula and the presence of PVR.

3. An important role in the development of PVR can be attributed to the chemokines involved in the late phase of wound healing.

Methods

Subjects

The present study was approved by the Hungarian Medical Research Council Committee of Science and Research Ethics (Approval No. 15028-2/2017/EKU) and performed in accordance with the tenets of the Declaration of Helsinki. All participants gave informed consent to the study.

Seventy-three eyes of 73 patients undergoing pars plana vitrectomy were included in the cross-sectional study. Patients were divided into four groups according to the indicated ocular pathology: 30 patients with RRD (without PVR), 16 patients with PVR, 8 patients with PDR and 19 control patients with idiopathic epiretinal membrane (ERM). The exclusion criteria were previous vitreoretinal surgery, penetrating injury, uveitis, aphakia, age-related macular degeneration, and uncontrolled glaucoma. Diabetes mellitus was excluded in the RRD, PVR and ERM groups. In the PDR group, only well-controlled diabetic patients (with glycated hemoglobin [HbA1c] under 8%) were included, and pure tractional RD was present (without vitreous hemorrhage or active proliferation). Previous panretinal photocoagulation was permitted, but intravitreal treatments (steroid or anti-VEGF) were excluded. The vitreous samples of patients with diabetic retinopathy, RD and ERM were collected for two years. As a limited number of samples can be analyzed on one kit of the multiplex chemiluminescent

immunoassay, we had to define the maximum number of vitreous fluids. After we analyzed the patients' data, we excluded the samples that were not matched the standing criteria. We had to exclude a lot of the diabetic samples because of previous intravitreal injections, active proliferation and high glycated hemoglobin.

In a subgroup analysis 58 eyes of 58 patients were studied. Indication for vitrectomy included macula off (n = 16) and macula on (n = 13) RRD, RRD with PVR (n=13) and ERM (n = 16). Patients who had a history of previous vitreoretinal surgery, penetrating injury, uveitis, aphakia, age-related macular degeneration, diabetic retinopathy, and uncontrolled glaucoma were excluded.

Vitreous sample preparation

Undiluted vitreous samples were collected during a standard three-port 23G pars plana vitrectomy by two surgeons (MR and TM). During core vitrectomy, vitreous fluid (0.5 ml) was collected from the eyes before starting irrigation. The samples were stored in Eppendorf tubes, cooled in a freezer at -20°C for some hours, and then frozen at −80°C until the assay was performed.

Cytokine analysis

Vitreous samples were analysed using a multiplex bead-based immunoassay, the Bio-Plex system (Bio-Rad Laboratories, Hercules,

CA, USA). Human Cytokine Screening Panel, 48-Plex (Bio-Rad Laboratories) was used to detect the molecules. The vitreous fluid was diluted fourfold through the use of sample diluent provided by the Bio-Plex beads array kit (Bio-Rad Laboratories, Hercules, CA). Dilution was chosen according to the relevant previous papers an expected range of concentrations. (Takahashi, Adachi et al. 2016) (Kunikata, Yasuda et al. 2013) Samples were prepared by first centrifuging the specimen at 10,000 ×g for 5 min after vortex agitation. A total volume of 50 µL from each sample was used for the assay. The kits were used according to the manufacturer's instructions by an experienced technician using the Bio-Plex 100 array reader with Bio-Plex Manager (software version 6.1); Bio-Rad Laboratories, Hercules, CA, USA).

Forty-eight molecules were measured, including cutaneous T-cell attracting chemokine (CTACK), eotaxin, basic fibroblast growth factor (basic FGF), granulocyte colony-stimulating factor (G-CSF), granulocyte–macrophage colony-stimulating factor (GM-CSF), growth-related oncogene alpha (GRO-alpha), hepatocyte growth factor (HGF), interferon alpha2 and gamma (IFN-alpha2 and IFN-gamma), IL-1alpha (interleukin), IL-1beta, IL-1 receptor antagonist (IL-1ra), IL-2, IL-2 receptor alpha (IL-2Ralpha), IL-3, IL-4, IL-5, IL -6, IL -7, IL -8, IL -9, IL -10, IL -12/p40, IL-12/p70, IL-13, IL -15, IL -16, IL -17, IL -18, interferon gamma–induced protein 10 (IP-10), leukemia inhibitory factor (LIF), monocyte chemotactic protein 1 and 3 (MCP-1, MCP-3), macrophage colony-stimulating factor (M-CSF), macrophage migration inhibitory factor (MIF), monokine induced by interferon gamma

(MIG), macrophage inflammatory protein 1 alpha and beta (MIP-1alpha, MIP-1beta), beta-nerve growth factor (beta-NGF), platelet-derived growth factor (PDGF-BB), regulated upon activation, normal T cell expressed and secreted (RANTES), stem cell factor (SCF), stromal cell–derived factor 1alpha (SDF-1alpha), stem cell growth factor beta (SCGF-beta), tumor necrosis factor alpha and beta (TNF-alpha, TNF-beta), tumor necrosis factor–related apoptosisinducing ligand (TRIAL) and vascular endothelial growth factor (VEGF). The measurements of these cytokines were performed by using 96-well assay plates and reagent kits according to the procedure provided by the manufacturer.

Statistical analysis

After calculating the cytokine concentrations in the specimens, a Kruskal–Wallis analysis of variance and Dunn's multiple comparison test was performed, and P-values were calculated via dedicated statistical software (GraphPad Prism, La Jolla, CA). *P*-values <0.05 were set to indicate statistically significant results.

Results

Intraocular concentrations of cytokines, chemokines, and growth factors in RRD, PVR, and PDR

Seven cytokines had significantly higher concentrations in the case of all RD groups (RRD, PVR, and PDR) compared to controls: Levels of IL-6 ($p<0.001$, $p<0.001$ and $p<0.001$ respectively), IL-16 ($p<0.01$, $p<0.001$ and $p<0.001$ respectively), IFN-gamma ($p<0.001$, $p<0.001$ and $p<0.05$ respectively), MCP-1 ($p<0.001$, $p<0.001$ and $p<0.01$ respectively), MIF ($p<0.001$, $p<0.001$ and $p<0.001$ respectively) were significantly higher in all groups of RD compared to the group of ERM. The concentrations of IL-8 ($p<0.01$, $p<0.001$, and $p<0.01$ respectively) and eotaxin ($p<0.05$, $p<0.001$, and $p<0.01$ respectively) were significantly higher in PVR and PDR compared to ERM, and significantly lower in RRD compared to PDR.

There were four cytokines in PDR and PVR groups that had significantly higher levels compared to RRD and ERM **(Figure 6.)**: the level of CTACK was highly increased in patients with PVR ($p<0.05$ PVR vs RRD) and PDR ($p<0.01$ PDR vs ERM; $p<0.001$ PDR vs RRD). Levels of IP-10 were augmented in PDR and PVR vs ERM ($p<0.001$ both), increased in PDR vs RRD ($p<0.05$), but not different in PVR vs RRD. SCGF-beta exhibited the highest expression levels in PVR ($p<0.05$ PVR vs RRD), while not different in PDR vs ERM and RRD.

SDF1-alpha was prominent in the PVR ($p<0.001$ PVR vs ERM; $p<0.01$ PVR vs RRD) and the PDR ($p<0.001$ PDR vs ERM; $p<0.01$ PDR vs RRD) groups.

The concentration values of VEGF in the vitreous fluid were significantly higher in the PDR group ($p<0.05$ PDR vs ERM; $p<0.001$ PDR vs RRD and $p<0.01$ PDR vs PVR). The vitreous level of IL-18 was found to be elevated in the PDR group compared to ERM ($p<0.01$) and RRD ($p<0.05$).

Levels of IL-2Ralpha ($p<0.05$), IL-17 ($p<0.05$), and HGF ($p<0.05$) were significantly higher in PDR compared to RRD. The concentration of Beta-NGF was significantly elevated in PDR compared to RRD ($p<0.05$) and PVR ($p<0.05$). The levels of MIG were significantly higher in PDR ($p<0.01$) and ERM ($p<0.001$) compared to RRD.

Subgroup analysis and comparison of the intraocular concentrations of cytokines in eyes with PVR, macula on and macula off RRD

A total of 48 cytokines, chemokines, and growth factors were analysed in the vitreous samples and compared between the four groups. An assay could be performed on all samples. A Kruskal-Wallis test and Dunn's multiple comparison test selected 24 out of 48 cytokines, which reached the level of significance in concentration.

Levels of six molecules were higher in the case of all RD groups (PVR, macula off, and macula on RRD) compared to the control group. Levels

of HGF (p<0.0001), IFN-gamma (p<0.0001), IL-6 (p<0.0001), IL-16 (p<0.0001), MIF (p<0.0001), MCP-1 (p<0.0001) were significantly higher in all groups of RD compared to the group of ERM. The concentration of IL-8 (p=0.0003) was significantly higher in PVR and macula off RRD compared to the control group, but we could not find upregulation in macula on RRD. The concentrations of three molecules out of six were higher than 1 ng/ml in all RD groups (median concentrations in PVR: HGF= 8.135 ng/mL, MCP-1= 1.950 ng/mL, MIF= 4.371 ng/mL).

There were eight molecules that had significantly higher levels in PVR compared to macula on RRD and ERM: CTACK (p=0.0012), eotaxin (p=0.0006), G-CSF (p=0.0014), IP-10 (p<0.0001), MIG (p<0.0001), SCF (p=0.0001), SCGF-beta (p<0.0001), SDF-1alpha (p=0.0002). Levels of G-CSF and SCF were additionally significantly higher in macula off RRD compared to macula on RRD. The concentration of IP-10 was significantly higher in macula off RRD compared to ERM as well. SCGF-beta exhibited the highest expression levels in PVR group (median concentration= 31569 pg/mL). Levels of four out of eight molecules were higher than 100 pg/mL (median concentration in PVR: G-CSF= 129.9 pg/mL, IP-10= 958.1 pg/mL, MIG= 205.3 pg/mL, SDF-1alpha= 242.9 pg/mL).

Concentration of six molecules were significantly higher in PVR compared to macula on RRD: IL-1ra (p=0.0041), IL-5 (p=0.0035), IL-9

(p=0.0111), M-CSF (p=0.0114), MIP-1alpha (p=0.0046), TRAIL (p=0.0209).

We found that the concentrations of three molecules were significantly lower in macula on RRD compared to ERM: IL-1alpha (p=0.0202), IL-12(p40) (p=0.0174), IL2-Ralpha ($p<0.05$). The level of IL2-Ralpha was significantly higher in PVR compared to macula off and macula on RRD ($p<0.0001$) as well.

Conclusions

We conclude, that our results indicate that complex and significant immunological mechanisms are associated with the pathogenesis of different forms of RD such as RRD, PVR, and PDR. Concentrations of cytokines, chemokines, and growth factors are elevated in the vitreous of eyes with RD, the increase is dependent on the form of RD. The detected proteins are present in different concentrations both in RRD and PVR. In the presence of PVR and PDR, levels of the majority of cytokines are significantly elevated, thus they may serve as biomarkers to estimate the progression or severity level of proliferation. Our study adds new biochemical information to the previous studies in correlation with proliferative vitreoretinal alterations. The more exact knowledge of levels of vitreal cytokines may represent novel, therapeutic targets in the management of these diseases. Future investigations should focus on identifying the potential biomarkers to be able to intervene before irreversible proliferative alterations occur.

Intraocular concentrations of cytokines, chemokines, and growth factors in RRD, PVR, and PDR

To our knowledge, our reports are the first to simultaneously evaluate the concentrations of these 48 cytokines, chemokines, and growth factors in different forms of RD, including RRD, PVR, and PDR with tractional RD.

The concentration of seven cytokines was elevated in RD compared to controls: IL-6, IL-16, IFN-gamma, MCP-1, MIF. The concentrations of IL-8 and eotaxin were significantly higher in PVR and PDR compared to ERM, and significantly lower in RRD compared to PDR. Levels of CTACK, IP-10, SCGF-beta, and SDF-1-alpha were increased in PDR and PVR groups compared to RRD and ERM.
The concentration of VEGF and IL-18 were higher in PDR. Levels of IL-2Ralpha and HGF were higher in PDR compared to RRD. The concentration of Beta-NGF was significantly elevated in PDR compared to RRD and PVR. The levels of MIG were higher in PDR and ERM compared to RRD.

Subgroup analysis and comparison of the intraocular concentrations of cytokines in eyes with PVR, macula on, and macula off RRD

Furthermore, we are the first to publish that there is a difference in the cytokine pattern of the vitreous of patients with macula off and macula on RRD. In macula on RRD, the concentrations of 14 molecules were significantly lower compared to PVR. Significant differences were found between macula on and macula off RRD in the concentrations of G-CSF and SCF.

Comparison of the levels of intravitreal cytokines, chemokines, and growth factors of eyes in correlation with the position of the macula lutea (macula on, macula off RRD, and PVR).

Levels of HGF, IFN-gamma, IL-6, IL-16, MIF, and MCP-1 were increased in the case of all RD groups compared to the control group. The concentration of IL-8 was higher in PVR and macula off RRD compared to the control group, but not in macula on RRD.

In PVR compared to macula on RRD and ERM: CTACK, eotaxin, G-CSF, IP-10, MIG, SCF, SCGF-beta, SDF-1alpha were elevated. Levels of G-CSF and SCF were elevated in macula off RRD compared to macula on RRD. The concentration of IP-10 was significantly higher in macula off RRD compared to ERM as well.

In PVR compared to macula on RRD: concentrations of IL-1ra, IL-5, IL-9, M-CSF, MIP-1alpha, TRAIL and IL2-Ralpha were higher.

Concentrations of IL-1alpha, IL-12(p40), and IL2-Ralpha were significantly lower in macula on RRD compared to ERM. The level of IL2-Ralpha was significantly higher in PVR compared to macula off and macula on RRD.

Hypotheses:

Our data supported all our hypotheses.

Patients with macula off RRD and PVR have higher levels of cytokines compared to patients with macula on RRD.

There is a correlation between intravitreal cytokine expression and the position of the macula and the presence of PVR.

Concentrations of 15 out of 48 cytokines were significantly higher in PVR compared to macula on RRD: CTACK, eotaxin, G-CSF, IP-10,

MIG, SCF, SCGF-beta, SDF-1alpha, IL-1ra, IL-5, IL-9, M-CSF, MIP-1alpha, TRAIL, and IL2-Ralpha.

Levels of G-CSF and SCF were significantly higher in macula off RRD compared to macula on RRD as well.

These elevated cytokines in PVR and macula off RRD compared to macula on RRD support the hypothesis that there is a correlation between intravitreal cytokine expression and the position of the macula and the presence of PVR.

An important role in the development of PVR can be attributed to the chemokines involved in the late phase of wound healing.
The concentrations of cytokines that are mainly present in the early phase were increased in all of the RD groups, but the level of IP-10 that participates in the proliferative and remodelling phase was higher only in the macula off RRD and PVR group. Our findings indicate that in the pathophysiology of PVR, those chemokines have a key role that participates in wound healing, especially in the late phase.

Bibliography of the candidate's publications

Thesis related publications

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