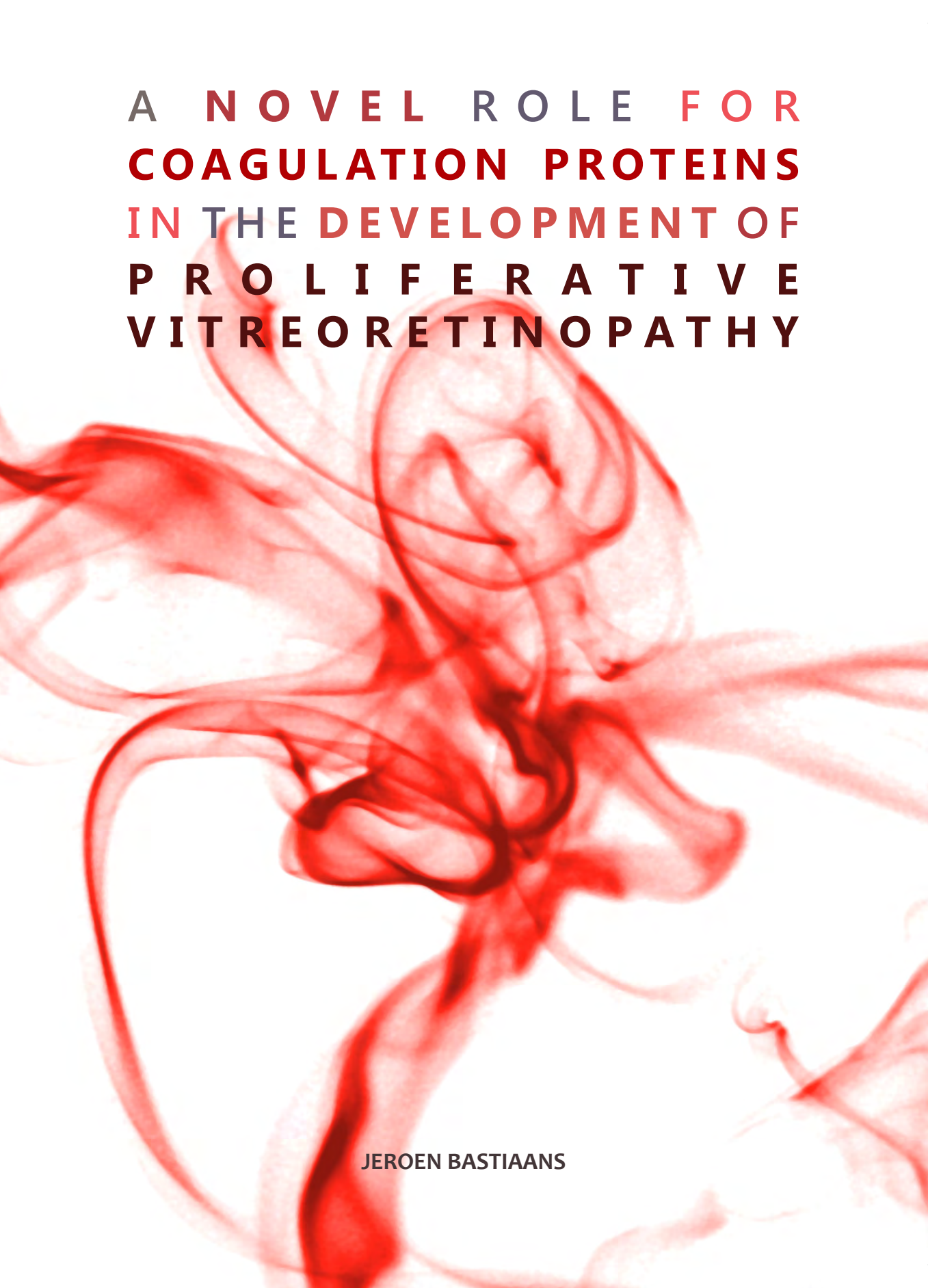
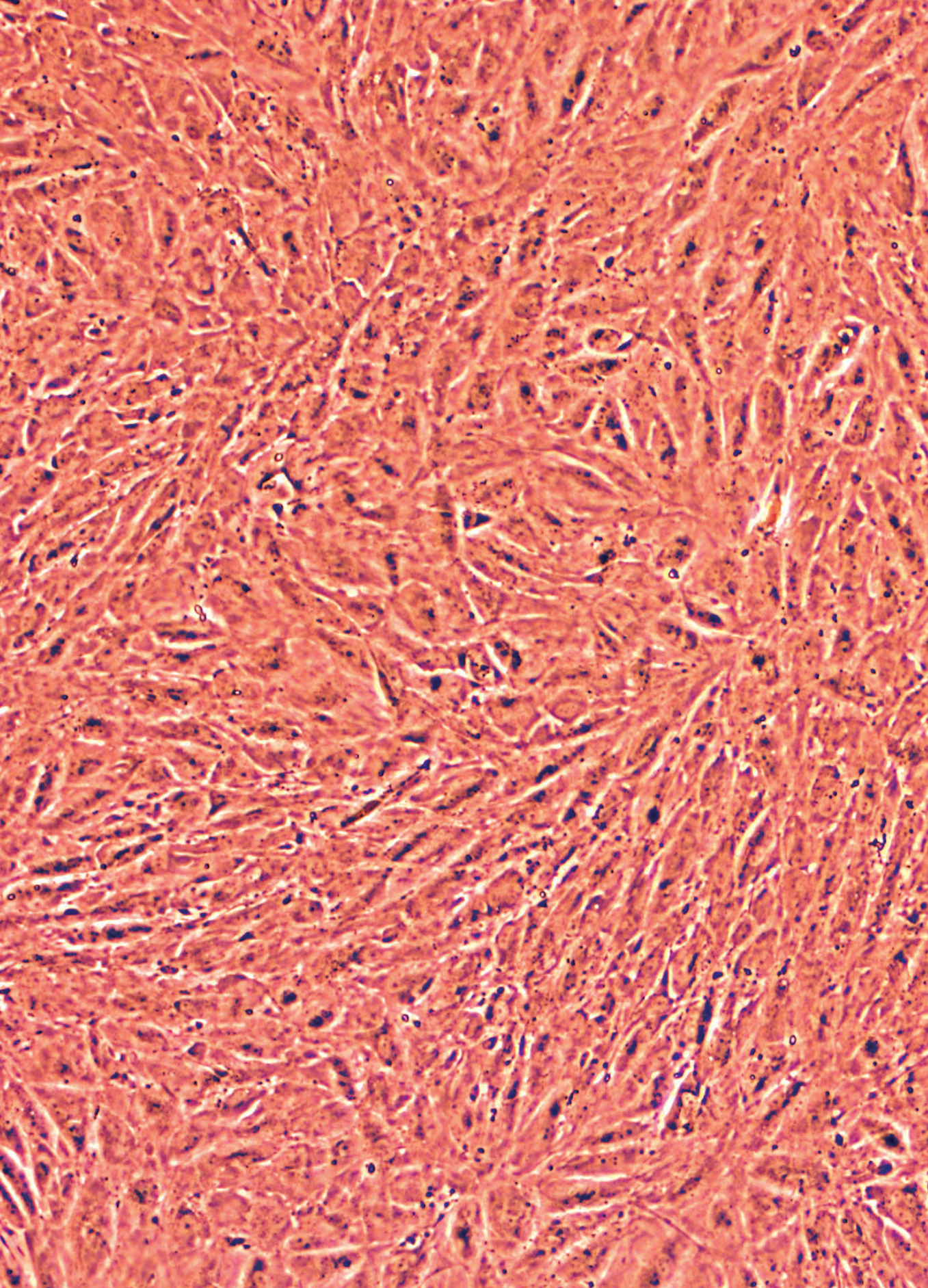
A NOVEL ROLE FOR COAGULATION PROTEINS IN THE DEVELOPMENT OF PROLIFERATIVE VITREORETINOPATHY



JEROEN BASTIAANS



A NOVEL ROLE FOR COAGULATION PROTEINS IN THE DEVELOPMENT OF PROLIFERATIVE VITREORETINOPATHY

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The research for this thesis was performed within the framework of the Erasmus Postgraduate School Molecular Medicine.

The studies described in this thesis were performed at the Department of Immunology, Erasmus University Medical Center, the Netherlands in collaboration with the Rotterdam Eye Hospital, the Netherlands.

The studies were financially supported by Combined Ophthalmic Research Rotterdam (CORR-Project code: 3.1.0) and the International Retinal Research Foundation, Birmingham, Alabama, United States.

The printing of this thesis was supported by the Erasmus University Medical Center, Ipsen Farmaceutica B.V., Landelijke Stichting voor Blinden en Slechtzienden, Rotterdamse Stichting Blindenbelangen, Stichting Blindenhulp and Stichting Ooglijders.

ISBN/EAN 978-94-6233-054-2

Illustrations: Sandra de Bruin-Versteeg and Jeroen Bastiaans
Cover-picture: Maaike de Bie and Peter de Bie
Lay-out: Schröder & Walters - creative services
Printing: Gildeprint Drukkerijen, Enschede, the Netherlands

A Novel Role for Coagulation Proteins in the Development of Proliferative Vitreoretinopathy

Een nieuwe rol voor stollingseiwitten in de ontwikkeling van proliferatieve vitreoretinopathie

Proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam op gezag van de rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties

de openbare verdediging zal plaatsvinden op
woensdag 23 september 2015 om 11.30 uur door

Jeroen Bastiaans

geboren te ‘s Hertogenbosch

Erasmus University Rotterdam

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The colors of the rainbow so pretty in the sky
Are also on the faces of people going by
I see friends shaking hands saying how do you do
But they're really saying I love you.

Louis Armstrong

Ter nagedachtenis aan mijn vader

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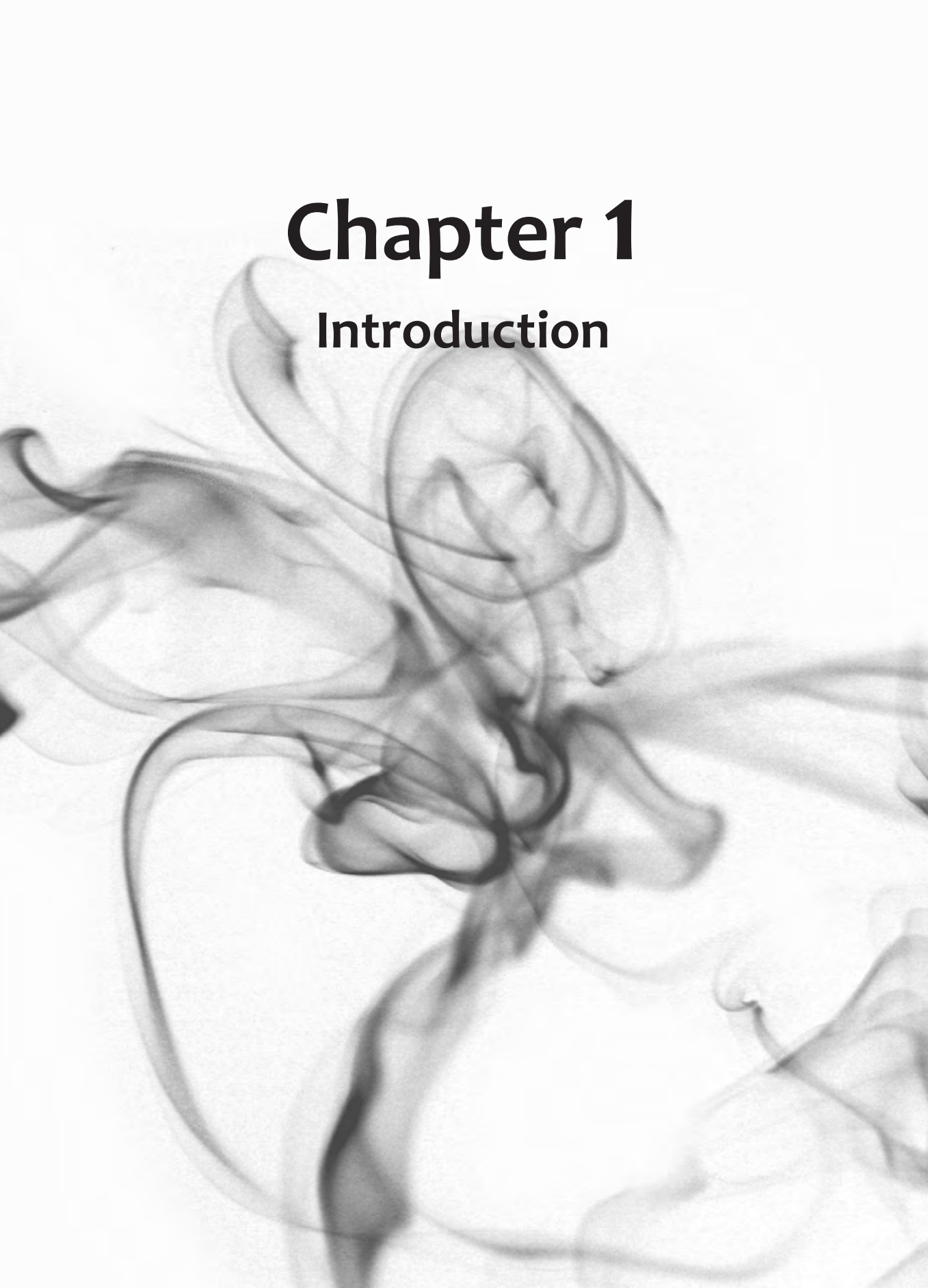
Chapter 1

Introduction



Proliferative vitreoretinopathy

Proliferative vitreoretinopathy (PVR) occurs in approximately 10% of patients with rhegmatogenous retinal detachment (RRD) and is the major cause of failed retinal detachment surgery [1-3]. Established PVR is characterized by the presence of subretinal, intraretinal, and/or epiretinal contractile fibroproliferative membranes that cause (re) detachment of the retina with reduced or even complete loss of vision as a consequence (Figure 1) [1-3].

Established PVR can currently only be treated by (further) surgical intervention with its potential risks. A pharmacological strategy to treat PVR would therefore be preferable. Current treatment approaches are anti-inflammatory agents (steroids), anti-proliferative agents (Daunomycin, 5-FU, retinoid acid) and heparin [4-8]. Although not clearly described in these studies, the likely rationale behind heparin therapy was to trap growth factors and prevent fibrin formation. However, only two of five studies involving heparin showed some benefit.

The pathophysiology of PVR comprises inflammation, cellular proliferation and deregulated tissue remodeling [1, 9, 10]. Although our knowledge on PVR development has increased during the last decades, more detailed insight into the cellular and molecular networks is absolutely required to identify and/or improve potential therapeutic strategies to prevent PVR in patients who are at risk. In order to investigate novel pathophysiological mechanisms we set up a series of experiments described in this thesis.

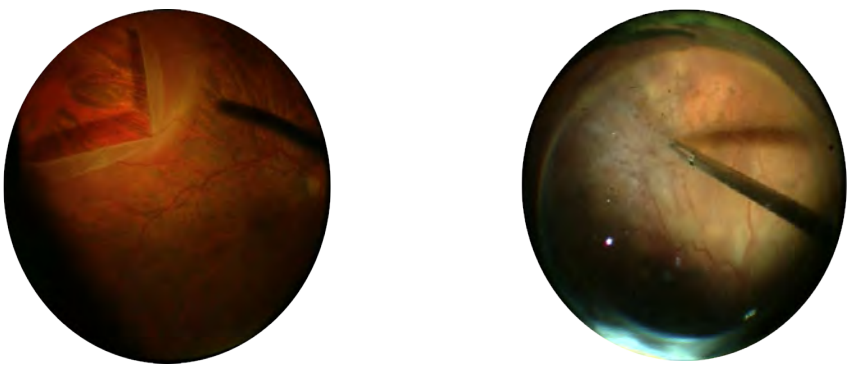


Figure 1: Fundus photographs of retinal detachments. Depicting a large retinal tear without fibroproliferative membranes (left) and an ablation with an epiretinal membrane (right).

Current insight in the pathophysiology of proliferative vitreoretinopathy

Inflammation and fibrosis represent central processes in the pathophysiology of PVR. Although far from completely understood PVR is the result of an intricate interplay between different cell types and soluble factors, including cytokines, growth factors, but also adhesion molecules.

Inflammation in PVR

Elevated levels of cytokines and chemokines, including chemokine (C-C motif) ligand (CCL)2, chemokine (C-X-C motif) ligand (CXCL)8, CXCL10, CXCL12, interleukin (IL)-6 and macrophage-colony stimulating factor (M-CSF), have been found in vitreous of patients with (established) PVR [11-19]. These cytokines/chemokines recruit, activate and stimulate the differentiation of immune cells at sites of disturbed tissue homeostasis, while some (e.g. IL-6) also enhance vascular permeability [20-22]. In line with this, increased vascular permeability is associated with PVR and leukocytes, primarily monocytes/macrophages, but also neutrophils, are present in vitreous and fibroproliferative membranes from patients with PVR, while B- and T-lymphocytes are present less frequently [1, 10, 23, 24]. The presumed contribution of the different types of leukocytes i.e. monocytes, macrophages, neutrophils and lymphocytes to PVR development is discussed hereunder.

Monocytes and macrophages

Upon tissue injury monocytes are recruited from the systemic circulation into the damaged tissue where they differentiate into macrophages that contribute to removal of cellular debris and, when present, infectious agents [25]. Instructed by the local environment, these macrophages can adopt either an M1 or M2-phenotype. The M1 phenotype is mostly associated with inflammatory responses. In contrast M2-macrophages secrete cytokines and growth factors, including IL-10, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF) that stimulate healing processes [26]. Unsuccessful healing attempts are associated with uncontrolled M2 skewing and consequently enhanced and prolonged production of growth factors [27]. Therefore, an important role is ascribed to M2-macrophages in fibrosis development [28-31]. Monocytes and macrophages are abundantly present in vitreous and retinal tissue from PVR patients [1, 10, 24]. Following retinal detachment macrophages can be expected to contribute to removal of cellular debris that originated from the damaging insult. However, they are also reported as important producers of pro-fibrotic mediators such as PDGF, transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF) that have been found elevated in vitreous from patients with PVR [1, 9, 10, 32]. Although to date no studies are available that examined M1 versus M2 skewing in PVR pathogenesis, M2-macrophages are likely to be involved in the fibrotic response associated with PVR. For example, activated autologous peritoneal macrophages induced PVR when injected into rabbit's eyes. These macrophages remained in the eye for a median period of 12 hours, a period probably sufficient to secrete the pro-fibrotic mediators mentioned above [33]. However, so far the mechanisms and molecules involved in monocyte recruitment and subsequent macrophage differentiation in PVR are still poorly understood, and are therefore not an attractive target for pharmacological intervention.

Neutrophils

Neutrophils have been identified in vitreous and retinal membranes from patients with PVR [10, 32, 34]. Their recruitment most likely involves the chemokine CXCL8 (IL-8), which is amongst the most potent neutrophil chemotactic factors and abundantly present in vitreous from PVR patients [34]. Despite their presence the contribution of neutrophils to PVR development is hardly examined as well. They can be expected to contribute to removal of cellular debris, but on the other hand might further aggravate tissue damage via the release of proteases including matrix metalloproteases that are elevated in PVR

ocular fluids or possibly other enzymes such as elastase [35-37]. Moreover, neutrophils are an extremely rich source of cytokines and as such can be involved in shaping the inflammatory environment [38].

Lymphocytes

B-lymphocytes and T-lymphocytes are only occasionally present in vitreous from patients with RRD, established PVR and in PVR membranes [10, 23, 24, 32, 39]. This suggests that lymphocytes only marginally contribute to PVR development. This is further supported by an experimental animal study in which PVR development and severity was compared between wild-type (WT) mice and RAG1-deficient mice (lacking B- and T-lymphocytes). This study demonstrated that although T-lymphocytes were present in PVR lesions of the WT mice, PVR incidence and severity was similar between WT and RAG1-deficient mice [40]. It can, however, not be excluded that lymphocytes, when present, contribute to the local inflammatory environment, for instance via the secretion of cytokines and growth factors such as IL-6 and TGF-β [34, 41].

Fibrosis in PVR

Fibrosis is often regarded as a deregulated healing attempt and is defined as excessive accumulation of extracellular matrix (ECM) components with an associated loss of normal tissue architecture, elasticity and function [42]. Although a variety of different insults can result in fibrosis the formation and proliferation of so-called myofibroblasts is a common feature of fibrosis in all organ system [43]. Myofibroblasts are highly specialized fibroblast-like cells that exhibit contractile properties and high capacity to synthesize ECM molecules [44]. Excessive ECM deposition and myofibroblast formation are evident histopathological features of PVR as well and will be discussed further below.

Extracellular matrix molecules

The fibrotic membranes in PVR contain a variety of ECM molecules that are normally absent or hardly present in the retina. The fibrillar collagen subtypes -I and -III are the most abundant, but also subtypes -II, -IV and -V have been detected in PVR membranes [45, 46]. Besides collagen, elastin, laminin, fibronectin and vitronectin have also been detected by immunohistochemistry in PVR membranes [47]. The ECM composition of retinal fibroproliferative membranes can vary in the course of disease. For instance, fibronectin appears to be more abundant in membranes of short clinical duration while collagen content increases in time [45, 48, 49]. Moreover, the cellularity of the membranes decreases with clinical duration [9, 47]. These changes in ECM composition and cellularity are comparable to those in healing and fibrosis in other organ systems [50].

Retinal pigment epithelial cells and myofibroblasts

RPE cells form a monolayer between the photoreceptors and Bruch's membrane and are considered as a part of the outer blood-retinal barrier (BRB). Highly specialized tight junction molecules between the RPE cells provide them with their barrier function (Figure 2) [51-53]. RPE cells are in close contact with Bruch's membrane at the lateral side while their apical side is separated from the neuroretina by subretinal spaces [51-53]. One of the main functions of RPE cells is to regulate the ionic composition within the subretinal space, which provides the photoreceptors with an environment for proper functioning. The RPE cells also transport waste, water and nutrients from the subretinal space, and participate in the visual cycle [54-56]. The RPE cells are a major source of ocular angioregulatory proteins like the anti-angiogenic pigment epithelium-derived factor (PEDF) and pro-angiogenic VEGF and thus play an important role in maintaining the ocular angiogenic homeostasis through a balanced production of these positive and negative regulatory factors [57].

During pathology RPE cells can lose their physiological properties and contribute to local inflammatory and fibrotic responses. RPE express many receptors that can bind cytokines, growth factors, pathogens and other ligands, but are themselves a rich source of cytokines and growth factors as well [58, 59]. In PVR the RPE cells exert a clear pro-fibrotic role. RPE cells are abundantly present in PVR membranes and de-differentiate into myofibroblasts by a process referred to as epithelial mesenchymal transition (EMT) [1, 10]. EMT is associated with loss of typical epithelial features (e.g. tight junction molecules like zonula occludens and E-cadherin) while mesenchymal features (e.g. α-smooth muscle actin (α-SMA) and fibronectin expression) that promote migratory capacity, invasiveness and resistance to apoptosis are acquired [60-62]. Importantly, myofibroblasts exhibit a strong capacity to produce collagen molecules when stimulated with growth

factors, including PDGF and TGF-β [63, 64]. In principle EMT represents a normal physiological response to tissue injury to promote wound closure and tissue repair [62]. In normal healing processes EMT is tightly controlled, but in fibrosis EMT programs are not attenuated, resulting in persistent myofibroblast formation and excessive ECM accumulation [65].

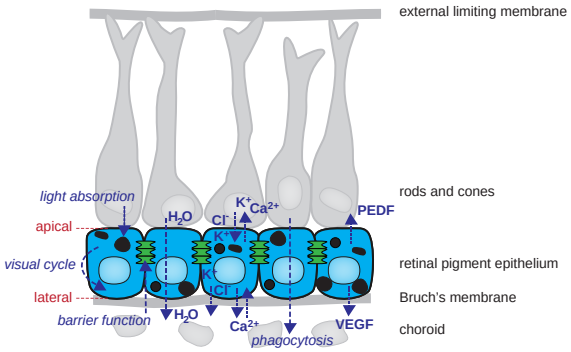


Figure 2: Schematic overview of the many functions of RPE in normal polarized condition: providing a protective barrier, light absorption, contribution to the visual cycle, phagocytosis, transepithelial transport of ions, nutrient secretion and immune modulation of the eye.

RPE-derived myofibroblasts thus represent an important source of the fibroblast-like cells that accumulate in PVR membranes and are indicative of the deregulated healing attempt [66]. Here myofibroblasts not only represent a major cell type involved in excessive ECM production, but the expression of α-SMA also provides these cells with contractile properties that cause PVR membrane contraction. This contraction contributes to further retinal detachment [45, 62, 67, 68]. These data point at an important role for EMT of RPE cells in the fibrotic process of PVR, however there is only limited data available on which factors exactly drive EMT by human RPE.

Other cell types

The retina contains astrocytes and glial cells, amongst which Müller cells that are the principal supporting glial cells of the retina [69-71]. Retinal damage/detachment induces proliferation of astrocytes and glial cells and immunohistochemical studies identified astrocyte and glial cell markers in PVR membranes [72]. These glial cells contribute to PVR membrane formation, mostly via collagen type-I production and myofibroblastic de-differentiation [73, 74].

In tissue repair processes (myo)fibroblasts originate from several sources, including the earlier discussed EMT. However at sites of tissue inflammation/healing fibroblast-like cells can also derive via recruitment and differentiation of circulating fibrocytes [75]. Fibrocytes are bone-marrow derived mesenchymal cells that circulate as peripheral blood mononuclear cells and express among others CD34, CD45, specific chemokine receptors as well as ECM molecules (e.g. collagen type-I and fibronectin) [75, 76]. Fibrocytes rapidly infiltrate sites of tissue damage where they participate in inflammation, healing and tissue remodeling, but they are also involved in fibrosis and as such they have been suggested to contribute to PVR development [77, 78]. However, how fibrocyte recruitment and subsequent differentiation is regulated in PVR requires further studies.

Growth factors in PVR development

Despite the variety of insults associated with fibrosis it is generally accepted that fibrosis is largely driven by excess presence of cytokines, growth factors and their receptors within the affected tissue where they stimulate (myo)fibroblast recruitment, proliferation, differentiation and ECM molecule production [79]. In PVR the true initiating insult remains enigmatic, but certain gene polymorphisms, for instance in mouse double minute 2, human homolog of; p53-binding protein, (MDM2), tumor protein (TP)53, mothers against decapentaplegic homolog (SMAD)7 and tumor necrosis factor (TNF) have been linked to PVR development, although their exact contribution remains to be established [80-83]. However, enhanced intraocular growth factor activity is clearly implicated in PVR pathogenesis [84-86]. These growth factors derive from different sources. They may for instance originate from blood secondary to BRB breakdown or via local production by the various described cell types including glial cells, RPE cells, (myo) fibroblasts and infiltrated immune cells [1, 10, 68, 84-86]. A variety of growth factors, their receptors and signaling pathways have been found elevated in vitreous and membranes from PVR patients and are summarized in table 1 [1, 87]. Moreover, connective tissue growth factor (CTGF), which binds domains of growth factors and matrix metalloproteases, is downstream of various growth factors amongst which PDGF, TGF-β and VEGF signaling, is also associated with PVR [84, 88-92]. CTGF contributes to physiological wound healing and fibrosis by inducing ECM production [88, 91, 92]. CTGF is highly expressed in human PVR membranes and partially co-localized with cytokeratin-positive RPE cells [89]. Furthermore a prominent accumulation of the N-terminal half of CTGF was found in the vitreous of patients with PVR [89]. Because PDGF, TGF-β and VEGF are thought to be amongst the most important growth factors involved in PVR they are discussed in more detail.

Platelet-derived growth factor

PDGF is a family of polypeptides that exert broad functions, both in health and disease [128]. The PDGF family consists of the dimeric family members, PDGF-AA, -AB, -BB, -CC and –DD. PDGF-AA and PDGF-BB are secreted in their active form after intracellular processing, while PDGF-CC and PDGF-DD are secreted as latent molecules that require extracellular activation by proteases [129]. PDGF molecules activate specific PDGF-receptor (PDGFR) dimers, being PDGFR-αα, PDGFR-αβ or PDGF-ββ dimers (table 1) [114, 130]. PDGF is not only released by platelet degranulation following injury but is also produced by a variety of cell types, including macrophages, fibroblasts and RPE cells [110, 131]. PDGF molecules stimulate chemotaxis and proliferation of different cell types including neutrophils, macrophages, fibroblasts, fibrocytes and smooth muscle cells at sites of injury [132, 133]. PDGF also induces myofibroblast differentiation and stimulates ECM production [134, 135]. PDGF-molecules are generally considered as key driving forces in fibrosis [136].

Of all growth factors measured to date, PDGF isoforms are amongst the ones most highly present in vitreous of patients and experimental animals with PVR, suggesting that they strongly contribute to disease pathogenesis [74, 89, 134]. The PDGF molecules can be blood derived or originate from local production by glial cells, RPE cells and macrophages [14, 137, 138]. PDGF induces proliferation and myofibroblast differentiation by glial cells and RPE cells [113]. Retinal glial cells express PDGFR-α and PDGFR-β chains, while RPE cells express the PDGFR-β chain at far higher levels than the PDGFR-α chain [114]. In human vitreous samples as well as in experimental animal models PDGF-CC has been identified as

important contributor to PVR development [139, 140]. However the clear involvement of RPE cells in PVR pathogenesis along with their predominant expression of PDGFR-β chains suggests a role for PDGF-BB and PDGF-DD that predominantly activate PDGFR-ββ dimers. Nevertheless the low expression of PDGFR-α chains by RPE does not exclude that PDGFR-αβ dimer activation by PDGF-AB, PDGF-BB, PDGF-DD, and possibly PDGF-CC, or PDGFR-αα dimer activation by PDGF-AA, PDGF-AB and PDGF-CC contribute to RPE activation in PVR [128]. PDGF-BB has been shown stimulate EMT by RPE via PDGFR signaling and PDGFR activity has been observed in human epi- and subretinal membranes, although this was not confined to a specific cell type [114, 141-143].

Table 1: Growth factors implicated in PVR

Growth factor	Growth factor receptor	Target cells	Cellular effect
epidermal growth factor (EGF)	EGF-receptor	glial cells [93-95] RPE [96, 97]	differentiation [98] migration [97]
fibroblast growth factor (FGF)	FGF-receptor	glial cells [99-101] RPE [99]	differentiation [102] migration [102] proliferation [101, 102]
heparin-binding EGF-like growth factor (HB-EGF)	EGF-receptor	glial cells [93-95] RPE [96, 97]	chemotaxis [95] differentiation [95] migration [97] proliferation [95]
insulin-like growth factor (IGF)	IGF-receptor insulin receptor	glial cells [103] RPE [104-107]	migration [104-106] proliferation [104, 106, 107]
pigment epithelium derived– factor (PEDF)	PEDF-receptor	RPE [108, 109]	anti-migration [108, 109]
platelet-derived growth factor (PDGF)	PDGF-receptor	fibroblast [110, 111] glial cells [103] RPE [110 112-114]	chemotaxis [112, 113] differentiation [115] ECM production [116] proliferation [112]
transforming growth factor – α (TGF-α)	EGF-receptor	glial cells [93-95] RPE [96, 97]	differentiation [98] migration [97]
transforming growth factor – β (TGF-β)	TGF-β-receptor	fibroblasts [117] RPE [118-120]	differentiation [117, 118, 120, 121] ECM production [88, 119] proliferation [118]
vascular endothelial growth factor (VEGF)	VEGF-receptor	glial cells [122] RPE [122, 123]	proliferation [124]
hepatocyte growth factor (HGF)	c-MET	RPE[125, 126]	proliferation [125-127] migration [125, 127]

Transforming growth factor-β

Transforming growth factor (TGF)- β contains three isoforms, TGF-β_1, TGF-β_2 and TGF-β_3 TGF-β molecules are dimeric molecules that are initially secreted by the producing cells as a large latent complex that also contains two latency associated peptide (LAP) chains and a latent TGF-β binding protein (LTBP) [144]. After secretion active TGF-β is released from the latent complex, mainly via the action of proteases for instance plasmin, matrix metalloproteases (MMP)-2 and -9, bone morphogenetic protein (BMP)-1, but also other molecules such as thrombospondin 1, retinoic acid, αV integrins as well as reactive oxygen species and by an acidic environment (e.g. low pH) [145-149]. After formation the active TGF-β molecules interact with a receptor complex forming a heterotetrameric combination containing two type-I TGF-β-receptors (TGF-βR-I) and two TGF-βR-II subunits [150]. Besides TGF-βR-I and TGF-βR-II also TGF-βR-III is expressed. TGF-β-R-III is not a typical receptor as it is not able to transmit signaling by itself, but has a co-receptor function that can present TGF-β to a receptor complex consisting of TGF-βR-I and TGF-βR-II subunits [151-154]. TGF-β isoforms regulate cellular proliferation, differentiation, migration, survival, ECM production and are involved in embryonic development, angiogenesis, and wound healing [65, 155]. Besides this, excessive TGF-β activity is a major contributor to fibrosis development [156-158].

Increased expression of TGF-β and TGF-βR has been detected in epiretinal membranes of patients with PVR [66, 159, 160]. TGF-β has been demonstrated to induce proliferation and EMT by RPE [118, 161, 162]. RPE express TGF-β_1 and TGF-β_2 of which TGF-β_2 is the dominantly expressed isotype, and TGF-β_2 is most often associated with PVR [14, 67, 120, 163]. The importance of the TGF-β signaling pathway in PVR is further supported by in vitro and experimental animal models, in which TGF-β or downstream signaling was inhibited, which prevented proliferation and EMT of RPE [164-167].

Vascular endothelial growth factor

In humans four VEGF isoforms are presented known as VEGF-A, -B, -C and -D [168]. These isoforms signal through their receptors named VEGFR1, -2 and -3. VEGF-A binds VEGFR1 and -2, VEGF-B binds VEGFR1, VEGF-C binds VEGFR2 and -3 while VEGF-D binds VEGFR3 [169]. VEGFRs are distantly related to the PDGFR family but are unique with respect to their structure and signaling system. Signaling via VEGFR1 and VEGFR2 results in proliferation and migration of endothelial cells which is primarily involved in angiogenesis and increased vascular permeability, signaling via VEGFR3 stimulates lymphangiogenesis [170]. In several vitreoretinal disorders intraocular VEGF concentration levels are elevated [87, 171, 172]. In exudative age-related macular degeneration and (proliferative) diabetic retinopathy the elevated levels of VEGF largely contribute to vascularization of the retina [173-175]. VEGF is also abundantly present in vitreous and retinal tissue from patients with established PVR [1, 87, 172, 176, 177]. However, retinal vascularization is not part of PVR pathogenesis. In RPE VEGF induces PEDF expression which is an anti-angiogenic factor which competes with VEGF for VEGFR binding and thus inhibits VEGFR signaling [178]. Moreover, in an animal PVR model in which VEGF was neutralized by Ranibizumab, membrane formation was reduced [179]. VEGF stimulates the local fibrotic response in PVR via VEGFR-mediated transactivation of other growth factor receptors, including PDGFR-α [87, 123, 180].

Coagulation cascade activity in PVR

Coagulation cascade activation after tissue injury is crucial to facilitate the healing process. The coagulation cascade consists of a series of plasma proteins that are stepwise activated with the formation of a fibrin clot as final product. Two pathways of coagulation cascade activation are recognized, the intrinsic pathway and the extrinsic pathway, of which the extrinsic pathway (activated by vascular injury) is considered crucial for the initiation of fibrin formation while the intrinsic pathway is more involved in maintenance of fibrin formation (Figure 3) [181]. At a certain point activation of the coagulation cascade results in the conversion of factor X into activated factor X (FXa). FXa then cleaves pro-thrombin to generate thrombin which converts soluble plasma fibrinogen into an insoluble fibrin clot, but also stimulates platelet aggregation and degranulation [182]. The formed clot prevents blood from leaving the vessels and prevents pathogens from entering the blood stream [183]. In addition the fibrin/platelet clot provides a scaffold for fibroblasts, macrophages and other cells to migrate on, thereby facilitating the healing process. Once the site of injury is closed, the formed clot is degraded by plasmin in a process referred to as fibrinolysis and normal blood flow is re-established [183].

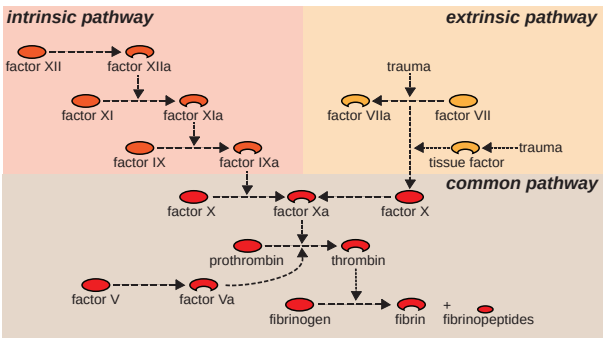


Figure 3: Schematic overview of the coagulation cascade. Both the intrinsic and the extrinsic pathway end up in the common pathway in which factor Xa converts prothrombin into thrombin that converts fibrinogen into fibrin.

Uncontrolled activation of the coagulation cascade has been recognized to contribute to fibrosis in several organ systems [184, 185]. Classically it was thought that this was related to the fibrin clot acting as matrix for fibroblasts and inflammatory cells to migrate and proliferate on as well as providing a reservoir function for binding and release of growth factors and cytokines [186]. Along this line, fibrin deposits have been found in the eyes of PVR patients and it has been reported that RPE can use fibrin as scaffold supporting de-differentiation and migration [9, 187]. However, the observation that fibrinogen knock-out mice were not protected from bleomycin-induced lung fibrosis questions whether fibrin formation is truly a prerequisite for fibrosis to develop [188].

FXa and thrombin, however, also directly activate cellular processes associated with healing through specific activation of proteinase-activated receptors (PARs), of which four different types exist: PAR1-4 [189-191]. PARs are G-coupled receptors of which signaling is initiated via proteolytic cleavage of the extracellular N-terminal part. The newly formed N-terminus binds to another extracellular part of the receptor, causing a conformational change that activates intracellular signaling via the G-coupled proteins (Figure 4) [192]. Each PAR has its own unique cleavage site that can be cleaved by various proteases of which thrombin by far has the highest affinity for these receptors, with the exception of PAR2 (Table 2) [191, 193, 194].

PAR1 represents the major signaling receptor for thrombin and FXa to induce pulmonary fibroblast proliferation, cytokine/growth factor and ECM production, as well as myofibroblast differentiation [195]. Importantly, direct thrombin or FXa inhibition protects mice from bleomycin-induced lung fibrosis [196, 197]. Moreover, PAR1 knock-out mice are protected from bleomycin-induced lung inflammation and fibrosis [196, 198]. Comparable observations have been made in animal models for fibrosis in other organs, for instance in the kidney and liver [185]. Together these data suggest that FXa/thrombin induced PAR1 signaling elicits cellular responses crucial for fibrosis to develop.
RPE cells also express PARs, mostly PAR1 and PAR3 [199]. However to date only minimal data is present that these cells can be activated by thrombin and FXa. Thrombin and FXa have been described to induce expression of VEGF, FGF, PDGF and TGF-β as well as chemotaxis and proliferation of human RPE cells [199-203]. In animal studies, with primary rat RPE, thrombin was described to induce stress fiber formation, proliferation, expression of adhesion molecules and increased expression of chemokines like CCL2 [204-207]. These observations together with the implication that the coagulation cascade is involved in PVR pathogenesis warrants further studies into the RPE activating effects of FXa and thrombin, especially because clinical applicable thrombin inhibitors are currently available.

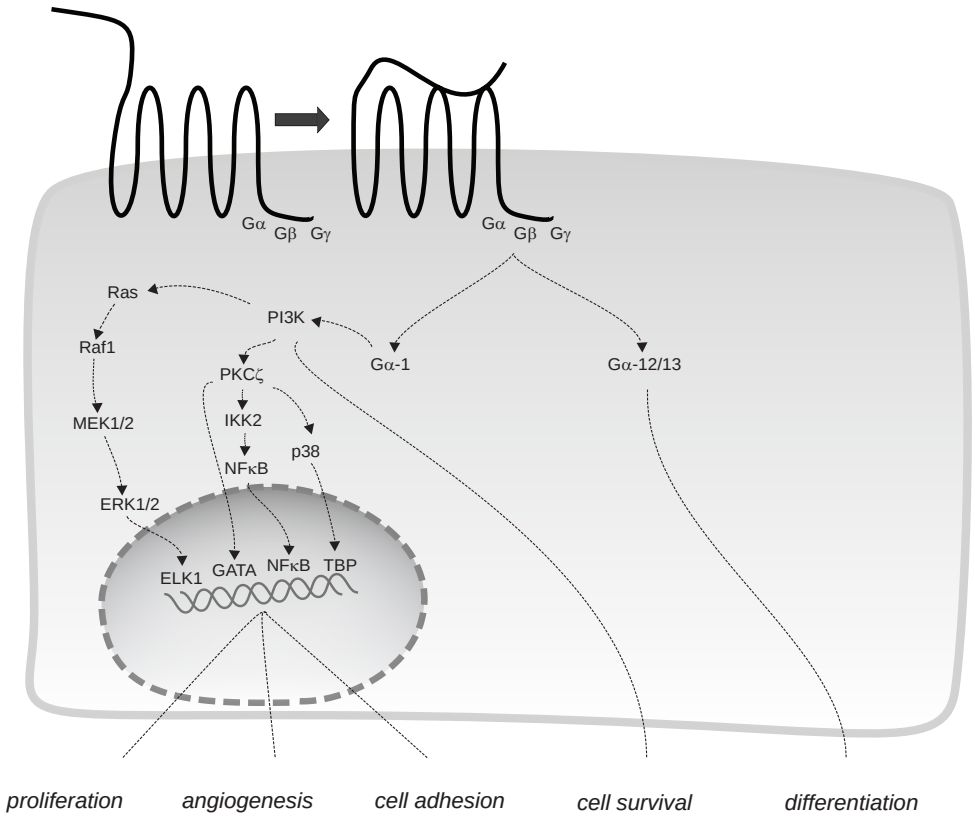


Figure 4: Schematic overview of PAR1 signaling which resulting in various cellular effects like cell growth, differentiation, inflammation and migration.

Table 2: Agonists of protease-activated receptors

PAR1	PAR2
Activated protein C Cathepsin G Factor Xa Granzyme A Kallikrein 1, 4, 5, 6, 14 Matrix metalloprotease 1 Meizothrombin (desF1) Penicillium citrinum 13 Plasmin Proatherocytin *Thrombin* Trypsin IV	Acrosin Bacterial gingipains Chitinase Granzyme A Kallikrein 2, 4, 5, 6, 14 Mast cell tryptase Matriptase/Membrane-Type Serine Protease 1 Penicillium citrinum 13 Peptidase 1, 2, 3 Tissue factor:Factor Xa:Factor VIIa Transmembrane protease serine 2 Transmembrane protease serine 11D Trypsin Trypsin IV
PAR3	**PAR4**
Thrombin	Bacterial gingipains Cathepsin G Factor Xa Kallikrein1, 14 Mannon binding lectin serine peptidase 1 Plasmin *Thrombin* Trypsin Trypsin IV

Aim of this thesis

There is preliminary data that suggest involvement of the coagulation cascade in PVR, but at what stage of development and how this would contribute to PVR development is far from clear. The aims of this thesis are: a) to examine the effect of the coagulation proteins FXa and thrombin on pro-inflammatory and pro-fibrotic responses by human RPE cells, and b) to determine whether PVR development in patients is truly associated with increased intravitreal coagulation cascade activity, c) whether this activation of the intravitreal coagulation cascade contributes to RPE activation towards a fibrotic response and d) if the latter process can be blocked by the clinically available direct thrombin inhibitor dabigatran.

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Chapter 2

Factor Xa and thrombin stimulate proinflammatory and profibrotic mediator production by retinal pigment epithelial cells: a role in vitreoretinal disorders?

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Graefes Arch Clin Exp Ophthalmol. 2013 Jul

Abstract

Background: Vitreoretinal disorders, including proliferative vitreoretinopathy (PVR), proliferative diabetic retinopathy (PDR) and exudative age-related macular degeneration (AMD), are a major cause of visual impairment worldwide and can lead to blindness when untreated. Loss of blood-retinal barrier (BRB) integrity associated with vitreoretinal fibrin deposition, inflammation, fibrosis and neovascularization contribute to the pathophysiological processes in these disorders. Retinal pigment epithelial (RPE) cells are well recognized to contribute to vitreoretinal inflammation/fibrosis and are likely to encounter contact with coagulation factor upon loss of BRB integrity.

Methods: An extensive study was performed in which we examined the effect of factor Xa and thrombin on the production of a broad panel of cytokines/chemokines and growth factors by RPE cells. For this purpose we used the ARPE-19 cell line as well as primary RPE cells, a glass slide based array that allows simultaneous detection of 120 cytokines/chemokines and growth factors, ELISA and real-time-quantitative PCR. The involved signaling cascade was examined using specific inhibitors for protease activated receptor (PAR)-1, PAR-2 and nuclear factor kappa-B (NF-κB).

Results: Factor Xa and thrombin regulated the production of cytokines and growth factors (including GM-CSF, IL-6, IL-8, MCP-3, PDGF-AA, PDGF-BB, TIMP-1 and TGF-α) that fit well in the pathobiology of vitreoretinal disease. Blocking studies revealed that the effects were mediated via PAR1 induced NF-κB activation.

Conclusions: Our findings suggest that factor Xa and thrombin can drive vitreoretinal inflammation and fibrosis and should be considered as treatment targets in vitreoretinal disorders such as PVR, PDR and AMD.

Introduction

Vitreoretinal disorders, including proliferative vitreoretinopathy (PVR), proliferative diabetic retinopathy (PDR) and age-related macular degeneration (AMD), are visual impairing diseases that affect millions of people and are potentially blinding when left untreated [1]. The pathobiological process of these disorders comprises breakdown of the blood-retinal barrier (BRB) at some stage, with vitreoretinal inflammation, fibrosis in PVR, PDR and AMD, and neovascularization in AMD and PDR [1]. Treatment options are limited so far, anti-VEGF treatment can be beneficial in case of PDR and AMD while PVR treatment mostly still depends on (recurrent) surgical intervention [1, 2].

The retinal pigment epithelial (RPE) cell-layer (located between the neural retina and the choriocapillaries) controls, under normal physiological conditions, the integrity of the outer BRB [3]. However, in case of an injurious event RPE cells can turn into important drivers of ocular inflammation and neovascularization via the production of cytokines and growth factors [2, 4]. The production of these cytokines and growth factors can also contribute to retinal fibrosis by stimulating proliferation, migration and differentiation of RPE cells into extracellular matrix (ECM) protein producing myofibroblast-like cells [1].

Breakdown of the outer BRB is associated with vascular damage/leakage and activation of the coagulation cascade with resultant formation of vitreoretinal fibrin deposits [1, 5]. Factor Xa and thrombin are crucially important for fibrin formation but can also stimulate cellular responses involved in inflammation, tissue repair and fibrosis [6, 7]. These latter effects are established through activation of cell-membrane expressed protease-activated receptors (PARs), mainly PAR1 and PAR2 [8]. Factor Xa and thrombin most likely come into contact with RPE in vitreoretinal disease, but direct cellular effects of factor Xa and thrombin on human RPE cell activity are only marginally explored so far. Thrombin has been shown to stimulate migration, proliferation, IL-8 and monocyte chemotactic protein (MCP)-1 production by RPE and to facilitate RPE monocyte interactions [9, 10]. Furthermore, thrombin has been described to increase mRNA expression of the B-chain of the pro-fibrotic dimeric molecule PDGF and to stimulate VEGF production by human RPE cells [10, 11]. Data on the effect of factor Xa on RPE activity is even more scarce as it was only very recently reported that factor Xa can stimulate VEGF production by human RPE [12].

To date, no detailed data is present on the effects of factor Xa and thrombin on the production of a broad panel of inflammatory and pro-fibrotic mediators that can be involved in retinal inflammation and fibrosis. Therefore, we here investigated the effect of factor Xa and thrombin on the production of a total of 120 inflammatory and pro-fibrotic mediators by RPE cells, for which we used a human cytokine antibody glass-slide array. Several factors present on the antibody array were also examined by ELISA or real-time quantitative-PCR. In addition, by using antagonists for PAR1, PAR2 and an inhibitor of the transcription factor NF-κB the signaling pathway involved was examined.

This study is the first to demonstrate that factor Xa and thrombin may fulfill a comprehensive role in driving inflammation and fibrosis in vitreoretinal diseases, and may thus be attractive therapeutic targets.

Materials and methods

Reagents

The human retinal pigment epithelial cell line (ARPE-19) was obtained from ATCC (Manassas, VA, US). ARPE-19 cells were cultured in DMEM/HAM's F-12 1:1 medium (HyClone Logan, UT, US), containing 10% heat inactivated fetal calf serum (FCS) and penicillin/streptomycin (all from BioWhittaker, Verviers, Belgium), hereafter referred to as RPE medium. For experiments ARPE-19 cells between passage 23-30 were used. A primary RPE cell culture was established from a surplus eye globe from a donor after enucleation for corneal transplantation, as described previously [13]. For experiments primary RPE cells between passage 5-8 were used. Under the used culture conditions the ARPE-19 and primary RPE cells were not pigmented. Human factor Xa, thrombin and the NF-κB inhibitor SC-514 were obtained from Calbiochem (La Jolla, CA, US). PAR1 antagonist SCH79797 was obtained from Tocris Bioscience (Bristol, UK) and PAR2 antagonist ENMD-1068 was obtained from ENZO Life Sciences (Zandhoven, Belgium). IL-6 (lower limit of detection: 7.8 pg/ml) and IL-8 ELISA (lower limit of detection: 7.8 pg/ml) were obtained from Invitrogen (Paisley, UK), granulocyte macrophage colony stimulating factor (GM-CSF) (lower limit of detection: 15.6 pg/ml), MCP-3 (lower limit of detection: 15.6 pg/ml), TGF-β_1 (lower limit of detection: 31.3 pg/ml) and tissue inhibitor of metalloproteinase (TIMP)-1 ELISA (lower limit of detection: 31.3 pg/ml) were obtained from R&D Systems (Abingdon, UK).

Factor Xa and thrombin titration studies

ARPE-19 cells were seeded at 5 x 10^5 cells/well into six-well plates (Nunc; Roskilde, Denmark), serum-starved in RPE medium containing 1% FCS and allowed to adhere overnight. To determine the optimal concentration for further studies, ARPE-19 cells were initially stimulated for 24 hours with different concentrations factor Xa (0, 0.25, 0.5, 1.0 and 2.0 U/ml) and thrombin (0, 1, 5 and 10 U/ml) and the culture supernatants were analyzed for IL-6 and IL-8 concentrations by ELISA according to the manufacturer's instructions.

Multiplex detection of 120 factors related to inflammation and fibrosis

ARPE-19 cells were seeded 5 x 10^5 cells/well into six-well plates, serum-starved in RPE medium containing 1% FCS and allowed to adhere overnight and thereafter stimulated with factor Xa (1 U/ml) or thrombin (5 U/ml) for 24 hours. Culture supernatants of 4 identical stimulations were pooled and analyzed on a Quantibody ® Human Cytokine Antibody Array 2000 (RayBiotech Inc; Norcross, GA, US) according to manufacturer's instructions. This approach allows simultaneous detection of 120 cytokines/chemokines and growth factors per sample. Briefly, the pooled culture supernatants were incubated on glass slide arrays spotted with antibodies directed against different cytokines/chemokines and growth factors, followed by incubation with a secondary biotin-labeled antibody and a tertiary Cy3-labeled streptavidin. The arrays were scanned (Laser scanner: Tecan; Mechelen, Belgium) and the cytokine concentrations in the samples were calculated. Quantifying analysis was performed with ImaGene 8.0 (Biodiscovery Inc; El Segundo, CA, US). A heatmap was created of the factors which expression changed $\geq$ 2-fold within the linear range of the reference curves between any of the tested conditions (unstimulated, factor Xa stimulated and thrombin stimulated).

Messenger RNA expression analysis by real-time quantitative PCR

RPE cells were seeded 5 x 10^5 cells/well into six-well plates, serum-starved in RPE medium containing 1% FCS and allowed to adhere overnight. Subsequently the cells were incubated for 4 hours (which we found optimal in initial time-course experiments) in the presence or absence of factor Xa (1 U/ml) or thrombin (5 U/ml). RNA was isolated using Isol-RNA Lysis Reagent (5 Prime; Hamburg, Germany) and reverse transcribed into cDNA. Transcript levels of PDGF-A, PDGF-B, TGF-α, TGF-β_1 and insulin like growth factor binding protein (IGFBP)-3 were determined by RQ-PCR (7700 PCR system; Applied Biosystems [ABI], Foster City, CA, US). In addition the mRNA expression levels of all four PAR family members (PAR1, PAR2, PAR3, and PAR4) were determined in unstimulated RPE cells. Messenger RNA expression of the RPE cell specific protein cellular retinaldehyde-binding protein (CRALBP) was determined in RPE cells and the colon epithelial cell lines HT-29 and Caco-2 (kindly provided by dr. Ron Smits, Erasmus MC, The Netherlands). Expression levels of the analyzed gene transcripts were normalized to the control gene Abelson [14]. Primer and probe combinations used are listed in table 1.

Cytokine analysis by ELISA

RPE cells were seeded 5 x 10^5 cells/well into six-well plates, serum-starved in RPE medium containing 1% FCS and allowed to adhere overnight. Cells were subsequently stimulated with factor Xa (1 U/ml) or thrombin (5 U/ml) for 24 hours in fresh RPE medium containing 1% FCS. After stimulation supernatants were analysed for GM-CSF, IL-6, IL-8, MCP-3, TGF-β_1 and TIMP-1 by ELISA according to manufacturer's instructions. In order to determine total TGF-β_1 concentrations, inactive TGF-β_1 was activated by addition of a 1M HCl solution that was subsequently neutralized by adding a 1.2M NaOH/0.5M Hepes solution, according to the manufacturer's instructions.

Table 1: PCR primer and probe sequences

Gene	Sequence fwd primer (5`-3`)
ABL [14]	TGGAGATAACATCTAAGCATAACTAAAGGT
CRALBP	GGTGGACATGCTCCAGGATT
IGFBP-3 [52]	ACGCACCGGGTGTCTGA
PAR1	CGGCGGCTGCTGCT
PAR2	GGCCGCCATCCTGCTA
PAR3	GGCCTCCTGCTTCTGTTGC
PAR4	CTGCGTGGATCCCTTCATCT
PDGF-A [53]	CGGGGTCCATGCCACTAA
PDGF-B [54]	TCCCGAGGAGCTTTATGAGATG
TGF-α	GCCAGGCCTTGGAGAACAG
TGF-β_1	CGCGTGCTAATGGTGGAA

PAR and NF-κB inhibition studies

RPE cells were seeded at 5 x 10^5 cells/well into six-well plates, serum-starved in RPE medium containing 1% FCS and allowed to adhere overnight. Subsequently the cells were incubated with SC-514 (100 μM), SCH79797 (250 nM) or ENMD-1068 (100 μM) 60 minutes prior to stimulation with factor Xa or thrombin. Depending on the type of analysis the cells were stimulated for 4 or 24 hours with factor Xa or thrombin and the effects of the inhibitors were determined via RQ-PCR or ELISA. The concentrations SCH79797, ENMD-1068 and SC-514 used, were non-toxic to RPE cells as determined by lactate dehydrogenase (LDH) release (Roche, Mannheim, Germany) and microscopic appearance of the cells.

Statistical analysis

Data obtained with the cytokine array were considered of interest when a ≥ 2-fold change between any of the three culture conditions was measured. GM-CSF, IGFBP-3, IL-6, IL-8, MCP-3, PDGF-A, PDGF-B, TGF-α, TGF-β_1 and TIMP-1 mRNA or protein expression levels were analyzed using the paired Student's *t*-test. A *P-value* < 0.05 was considered significant.

	Sequence rev primer (5`-3`)	Sequence probe (5`-3`)
	GATGTAGTTGCTTGGGACCCA	CCATTTTTGGTTTGGGCTTCACACCATT
	TACCATGGCTGGTGGATGAA	TTCCCAGCCCGGTTCAAAGCCAT
	TGCCCTTTCTTGATGATGATTATC	CCCAAGTTCCACCCCCTCCATTCA
	GGGTTCCTGAGAAGAAATGACC	CCAGAATCAAAAGCAACAAATGCCACC
	GGGATGTGCCATCAACCTTA	CACCATCCAAGGAACCAGTAGATCCTCTAAA
	GGAACTCTTCAAAAGAATTTGGG	TGATACAAACAACTTGGCAAAGCCAACCTT
	CCTGCCCGCACCTTGTC	TACTACGTGTCGGCCGAGTTCAG
	GGGGCCAGATCAGGAAGTTG	AGCTTCCTCGATGCTTCTCTTCCTCCG
	CGGGTCATGTTCAGGTCCAAC	AGTGACCACTCGATCCGCTCCTTTG
	AATCTGGGCAGTCATTAAAATGG	ACGTCCCCGCTGAGTGACCCG
	AGAGCAACACGGGTTCAGGT	CCACAACGAAATCTATGACAAGTTCAAGCAGA

Results

The concentration-dependent effect of factor Xa and thrombin on IL-6 and IL-8 production

Factor Xa and thrombin induced IL-6 and IL-8 secretion by ARPE-19 after 24 hours of stimulation in a concentration dependent manner. Factor Xa induced maximal secretion of IL-6 and IL-8 at a concentration of ~1-2 U/ml, while the optimal level of stimulation for thrombin was at a concentration of ~5-10 U/ml (Figure 1). No significant difference was observed between 1 and 2 U/ml factor Xa for the level IL-6 and IL-8 production. Also no significant difference was observed between 5 and 10 U/ml thrombin with regard to IL-6 and IL-8 production. Therefore for further studies factor Xa was used at a concentration of 1 U/ml and thrombin at a concentration of 5 U/ml.

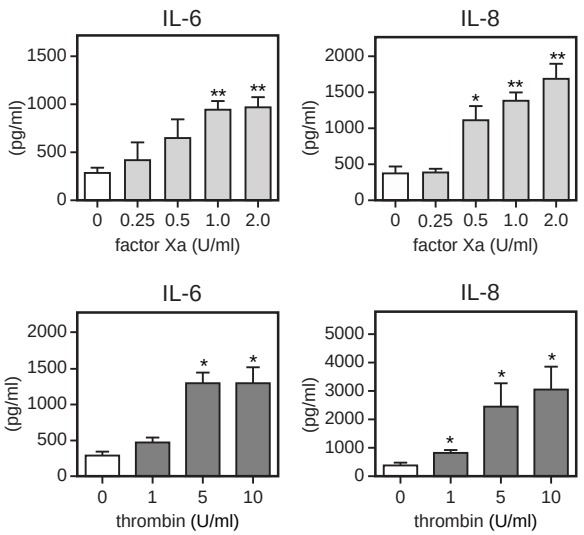

Figure 1: ARPE-19 cells were stimulated for 24 hours with different concentrations factor Xa or thrombin. IL-6 (left panels) and IL-8 (right panels) concentrations were determined in the culture supernatants by ELISA. Data are presented as the mean value of 4 independent experiments ± SEM. Statistical analysis was performed with the paired Student's *t*-test. $P < 0.05$ was considered significant. * = $P < 0.05$ and ** = $P < 0.01$, compared to unstimulated.

The effect of factor Xa and thrombin on a broad panel of cytokines and growth factors

Culture supernatants of factor Xa or thrombin stimulated ARPE-19 cells were analysed on quantitative human cytokine antibody arrays. All factors determined by cytokine antibody array analysis and the effect of factor Xa and thrombin on the production of these factors by ARPE-19 are given in supplemental table 1. The cytokine antibody array analysis revealed that factor Xa and thrombin both induced IL-6 and IL-8 (Figure 2A), confirming the data obtained by ELISA (Figure 1). Factor Xa further changed the expression of 8 other factors ≥ 2-fold; IGFBP-3, IL-9 and osteopontin (OPN) were downregulated compared to unstimulated cells, while GM-CSF, PDGF-BB, TIMP-1, tumour necrosis factor receptor (TNF-R)I and TGF-α were upregulated compared to unstimulated cells (Figure 2A). Thrombin induced a ≥ 2-fold change in expression levels for 10 factors other than IL-6 and IL-8; OPN was downregulated compared to unstimulated cells, while bone morphogenic protein (BMP)-7, GM-CSF, IL-17F, MCP-3 (CCL7), monokine induced by gamma interferon (MIG), osteoprotegerin (OPG), PDGF-AA, PDGF-BB, TIMP-1 and TNF-RI were upregulated compared to unstimulated cells (Figure 2A).

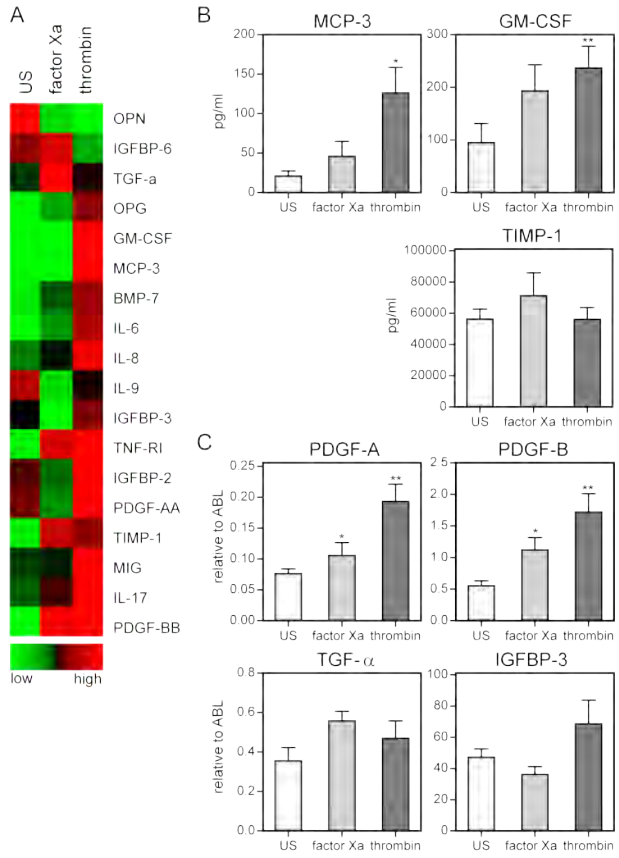

Figure 2: (A) ARPE-19 cells were stimulated for 24 hours with factor Xa (1 U/ml) or thrombin (5 U/ml). Culture supernatants of 4 identical stimulation experiments were pooled and analyzed on quantitative human cytokine antibody arrays. The heatmap represents cytokines and growth factors with ≥ 2-fold change between the culture conditions. (B) ARPE-19 cells were stimulated for 24 hours with factor Xa (1 U/ml) or thrombin (5 U/ml) and GM-CSF, MCP-3 and TIMP-1 concentrations in culture supernatants were determined by ELISA. Data are presented as the mean value from 4 independent experiments ± SEM. (C) ARPE-19 cells were stimulated for 4 hours with factor Xa (1 U/ml) or thrombin (5 U/ml) and IGFBP-3, PDGF-A, PDGF-B and TGF-α mRNA expression levels were determined by RQ-PCR and normalized against the control gene ABL. Data are presented as the mean value from 8 independent experiments ± SEM. US = unstimulated. Statistical analysis was performed with the paired Student's t-test. $P < 0.05$ was considered significant. * = $P < 0.05$ and ** = $P < 0.01$, compared to unstimulated.

To confirm the findings of the cytokine antibody array, a set of independent experiments was performed to examine the effect of factor Xa and thrombin on cytokine/chemokine/growth factor expression by ARPE-19 cells. For this purpose a selected set of factors for which ELISA systems and PCR primers and probes were available at our laboratory was used. In this independent set of experiments factor Xa significantly ($P < 0.05$) upregulated PDGF-A and PDGF-B mRNA expression by ARPE-19, while thrombin significantly ($P < 0.05$) upregulated the protein production of GM-CSF and MCP-3 as well as mRNA expression of PDGF-A and PDGF-B (Figure 2B). Also the pattern of IGFBP-3 and TGF-α mRNA expression and TIMP-1 protein production by ARPE-19 after stimulation with factor Xa and thrombin (Figure 2B) paralleled the findings obtained with the cytokine antibody array.

The effect of factor Xa and thrombin on TGF-β_1

The human cytokine antibody array analysis revealed that factor Xa slightly stimulated TGF-β_1 secretion (~1.5 times, supplemental table 1) and that thrombin had no effect. Because TGF-β_1 has been identified as an important stimulator of retinal fibrosis and the fact that the cytokine antibody array only detects TGF-β_1 in the active form, the effect of factor Xa and thrombin on total TGF-β_1 was also examined by RQ-PCR and ELISA. This showed that factor Xa and thrombin did not significantly enhance total TGF-β_1 production by ARPE-19 cells (Figure 3) [15].

Expression and activation of protease-activated receptors

Previously Hollborn and colleagues demonstrated that primary human RPE cells express PAR1 and PAR-3 mRNA [10]. To examine whether the ARPE-19 cells resemble this expression pattern the PAR mRNA expression profile was determined. This revealed that the ARPE-19 cells mainly express PAR1 and PAR3 with only marginal PAR2 and PAR4 expression (Figure 4A). ARPE-19 cells exhibit thus a PAR mRNA expression profile similar to that of primary human RPE and are therefore representative to study the effects of factor Xa, thrombin and PAR signaling in human RPE. Because factor Xa and thrombin mainly signal via PAR1 and PAR2 specific antagonists were used to examine which PAR receptor mediated their effects in RPE. PAR1 inhibition significantly reduced the capacity of factor Xa and thrombin to induce IL-6, IL-8, MCP-3 and PDGF-B production (Figure 4B and 4C). PAR1 inhibition also significantly reduced thrombin induced GM-CSF and PDGF-A and factor Xa induced TGF-α production. The PAR1 antagonist alone had no effect on basal production levels of these factors (data not shown). PAR2 blockade did not significantly alter factor Xa or thrombin induced production of GM-CSF, IL-6, IL-8, MCP-3, PDGF-A, TGF-α and TIMP-1 by ARPE-19 cells, nor did it affect basal production levels of these factors (data not shown), which is in line with the low PAR2 expression levels.

The effect of factor Xa and thrombin on NF-κB signaling

The transcription factor NF-κB initiates the transcriptional activation of many different cytokine/chemokine/growth factor genes [16]. Therefore we examined whether activation of the NF-κB signaling pathway was involved in mediating the effects of factor Xa and thrombin in RPE. The NF-κB inhibitor SC-514 significantly reduced factor Xa and thrombin induced production of the pro-inflammatory mediators IL-6, IL-8 and MCP-3 as well as thrombin-induced GM-CSF production by ARPE-19 (Figure 5A). Moreover, SC-514 significantly reduced factor Xa and thrombin induced production of the pro-fibrotic mediators PDGF-A and PDGF-B, as well as TGF-α (Figure 5B). Although factor Xa and thrombin marginally induced expression of the pro-fibrotic factor TIMP-1, it was consistently reduced by SC-514 (Figure 5B). SC-514 alone had no significant effect on the basal production of these factors (data not shown).

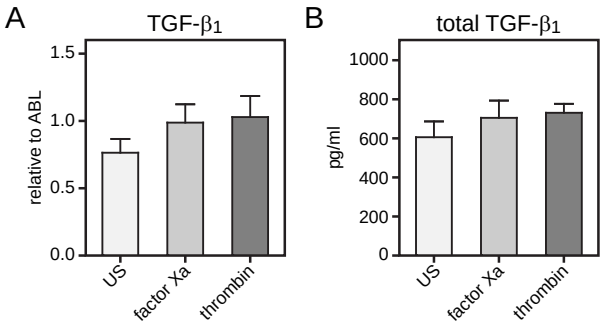

Figure 3: (A) ARPE-19 cells were stimulated for 4 hours with factor Xa (1 U/ml) or thrombin (5 U/ml) and the TGF-β_1 mRNA expression level was determined by RQ-PCR and normalized against the control gene ABL. Data are presented as the mean value from 4 independent experiments ± SEM. (B) ARPE-19 cells were stimulated for 24 hours with factor Xa (1 U/ml) or thrombin (5 U/ml) and total TGF-β_1 concentrations were determined in culture supernatants by ELISA. Data are presented as the mean value of 5 independent experiments ± SEM. US = unstimulated.

The effect of factor Xa and thrombin on primary RPE

The primary RPE cell culture established expressed the mRNA encoding the RPE specific protein CRALBP, comparable to ARPE-19 but in contrast to the colon epithelial cell lines HT-29 and Caco-2 that did not express CRALBP mRNA (Figure 6A), thus confirming their RPE origin. The primary RPE cells primarily expressed PAR1 and PAR3, thus resembling the PAR expression pattern of ARPE-19 (Figure 4A) and other primary RPE [10]. In line with the results found with ARPE-19, PAR1 inhibition significantly reduced factor Xa and thrombin induced expression of IL-6, IL-8 and PDGF-B in primary RPE, while this inhibition was non-significant for PDGF-A (Figure 6C and 6D). Also inhibition of the NF-κB pathway significantly reduced IL-6, IL-8, PDGF-A and PDGF-B expression induced by factor Xa or thrombin in primary RPE (Figure 6C and 6D).

Discussion

RPE cells contribute to vitreoretinal inflammation, fibrosis and neovascularization in disorders such as AMD, PDR and PVR [1, 2, 4]. Intraocular activation of the coagulation cascade is associated with vitreoretinal disease, and our study it the first that examined the effects of factor Xa and thrombin on the production of an extensive panel of inflammatory and pro-fibrotic mediators by RPE cells [1, 5]. We demonstrate that factor Xa and thrombin promote the production of several different cytokines, chemokines and growth factors by RPE cells and may thus play a comprehensive role in the inflammatory and fibrotic component of vitreoretinal (proliferative) disease.

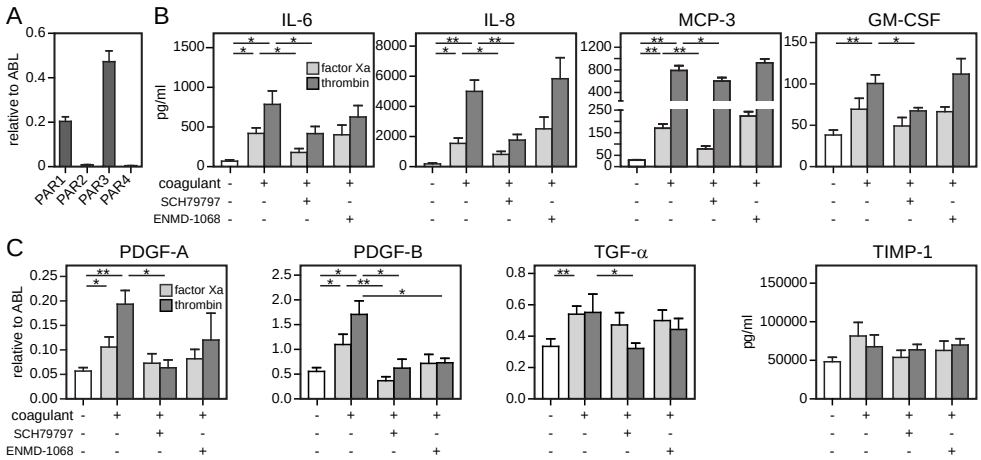

Figure 4: (A) PAR1, PAR2, PAR3 and PAR4 mRNA expression in ARPE-19 cells were determined by RQ-PCR and normalized against the control gene ABL. Data are presented as the mean value of 9 independent experiments ± SEM. ARPE-19 cells were stimulated for 24 hours with factor Xa (1 U/ml) or thrombin (5 U/ml) in the absence or presence of the PAR1 antagonist SCH79797 (250 nM) or the PAR 2 antagonist ENMD-1068 (100 μM). GM-CSF, IL-6, IL-8 and MCP-3 (B) and TIMP-1 (C) concentrations is culture supernatants were determined by ELISA. Data are presented as the mean value of 5 independent experiments ± SEM. (C) ARPE-19 cells were stimulated for 4 hours with factor Xa (1 U/ml) or thrombin (5 U/ml) in the absence or presence of the PAR1 antagonist SCH79797 or the PAR2 antagonist ENMD-1068 and the PDGF-A, PDGF-B and TGF-α mRNA expression levels were determined by RQ-PCR and normalized against the control gene ABL. Data are presented as the mean value of 5 independent experiments ± SEM. Statistical analysis was performed with the paired Student's t-test. $P < 0.05$ was considered significant. * = $P < 0.05$ and ** = $P < 0.01$.

To examine the effect of factor Xa and thrombin on the production of inflammatory and pro-fibrotic mediators by RPE we used a cytokine antibody array that simultaneously detects 120 cytokines/chemokines/growth factors. We also examined the effect of factor Xa and thrombin on the production of a set of factors identified with the cytokine antibody array by ELISA or RQ-PCR. This revealed a high level of concordance between expression patterns detected with the different techniques. From this we conclude that the cytokine antibody array is a reliable technique for multiplex analysis of culture supernatants and therefore several of the factors indentified with the cytokine antibody array technique will hereunder be discussed in relation to inflammation, fibrosis and vitreoretinal (proliferative) disorders.

Inflammation involves recruitment, activation and differentiation of immune cells, processes in which cytokines play a prominent role. Factor Xa and thrombin both stimulated the production of immune cell recruiting and activating cytokines GM-CSF, IL-6, IL-8, IL-17F, MCP-3 and MIG by RPE. IL-6 recruits and activates B-lymphocytes and stimulates plasma cell differentiation as well as immunoglobulin production, while IL-8 is powerful neutrophil chemoattractant [17, 18]. Thrombin also enhanced the production of IL-17F. The IL-17 family consists of distinct subclasses of cytokines: IL-17A-IL-17F, of which IL-17A and IL-17F share the most (~60%) homology. IL-17 stimulates the production of cytokines such as IL-8, thereby facilitating neutrophil recruitment [19]. MIG production was especially enhanced by thrombin and recruits NK cells and T-lymphocytes into inflammatory foci [20]. Thrombin also stimulated the production of MCP-3 and GM-CSF which are chemoattractants for monocytes and macrophages, while GM-CSF also stimulates differentiation of monocytes into macrophages [21]. B-lymphocytes, T-lymphocytes, monocytes, macrophages and neutrophils are abundantly present in infiltrates of AMD, PDR and PVR [2, 4, 22-27]. Collectively, our data demonstrate that factor Xa and thrombin induce cytokine/chemokine production by RPE and may thus play a role in inflammatory cell recruitment, activation and differentiation in vitreoretinal disease.

Inflammatory responses are generally controlled by counter regulatory anti-inflammatory molecules, such as soluble cytokine receptors and anti-inflammatory cytokines [28]. Thrombin stimulated OPG secretion by RPE while both factor Xa and thrombin enhanced TNF-RI secretion. OPG acts as decoy receptor for receptor activator of nuclear factor kappa-B ligand (RANKL) [29]. The RANK/RANKL/OPG system is well recognized for its role in bone metabolism, but is also involved in the regulation of inflammation and immunity. T-lymphocytes produce RANKL which stimulates RANK-expressing immune cells, such as monocytes/macrophages and dendritic cells, to secrete pro-inflammatory cytokines/chemokines [29]. OPG also acts as a soluble decoy receptor for TNF-related apoptosis inducing ligand (TRAIL) and can thereby prevent apoptosis induction [29]. Soluble TNF-RI acts as an anti-inflammatory cytokine that neutralizes TNF-α activity [30]. Thrombin and especially factor Xa downregulated IL-9 secretion by RPE. The functional activities of IL-9 are far from clear but it has been suggested to promote survival of for instance T-lymphocytes, mast cells, epithelial cells and neurons. Possibly improved survival under the influence of IL-9 might explain diverse IL-9 mediated effects such as enhanced chemokine expression by epithelial cells and antibody production by plasma cells [31]. The increased OPG and soluble TNF-R1 production by RPE upon thrombin stimulation and the decrease in IL-9 production upon factor Xa stimulation may thus represent anti-inflammatory mechanisms.

Recruitment, activation and differentiation of ECM-producing cells is central to the development of fibrosis and is driven by a host of mediators [32]. Our study demonstrates that factor Xa and thrombin activate RPE to produce several well recognized pro-fibrotic mediators. Factor Xa enhanced the production of TIMP-1 by RPE. TIMP's are the major endogenous inhibitors of ECM-degrading matrix-metalloproteinases (MMP), and decreased MMP-activity due to increased TIMP levels contribute to fibrosis [33]. In line with this, increased TIMP-1 levels have been described in PVR and correlated positively with disease severity [34]. The different PDGF isoforms stimulate migration, proliferation and ECM synthesis by various cell types, including epithelial cells and fibroblasts, thereby

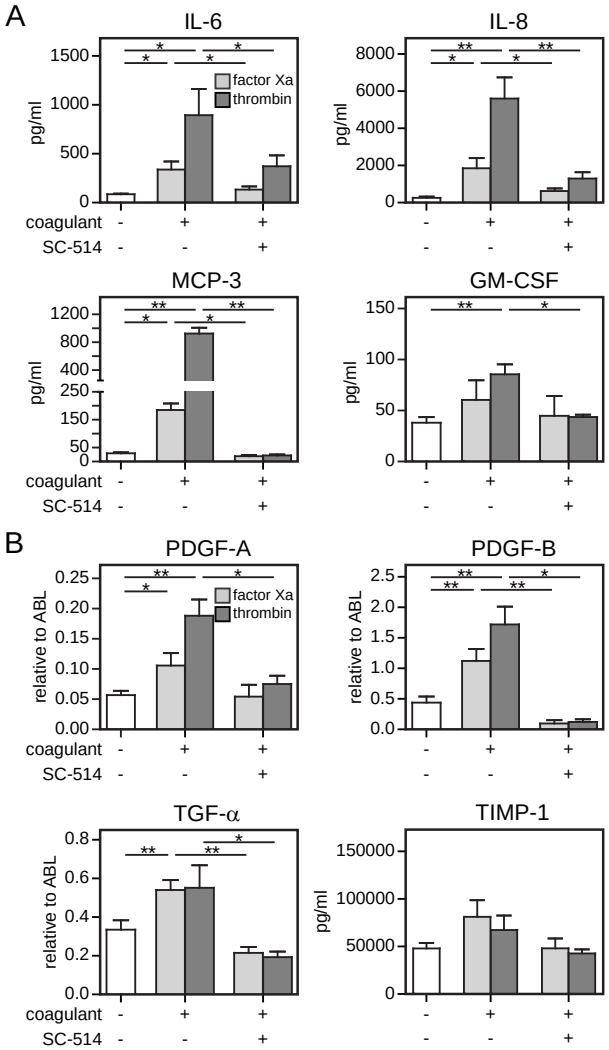

Figure 5: ARPE-19 cells were stimulated for 24 hours with factor Xa (1 U/ml) or thrombin (5 U/ml) in the absence or presence of the NF-κB inhibitor SC-514 (100 μM). GM-CSF, IL-6, IL-8 and MCP-3 (A) and TIMP-1 (B) concentrations in culture supernatants were determined by ELISA. Data are presented as the mean value of 4 independent experiments ± SEM. (B) ARPE-19 cells were stimulated for 4 hours with factor Xa (1 U/ml) or thrombin (5 U/ml) in the absence or presence of the NF-κB inhibitor SC-514 (100 μM). PDGF-A, PDGF-B and TGF-α mRNA expression levels were determined by RQ-PCR and normalized against the control gene ABL. Data are presented as the mean value of 5 independent experiments ± SEM. Statistical analysis was performed with the paired Student's t-test. $P < 0.05$ was considered significant. * = $P < 0.05$ and ** = $P < 0.01$.

contributing to fibrosis [35]. PDGF and (activated) PDGF-receptors have been found in epiretinal membranes from patients with proliferative retinopathies and PDGF receptor inhibition reduces experimental PVR [34, 36-39]. Our results demonstrate that thrombin and factor Xa collectively stimulate the formation of the pro-fibrotic PDGF-AA, PDGF-AB and PDGF-BB dimers by RPE cells. The stimulatory effect of thrombin on PDGF-B mRNA expression by RPE is in line with previous observations [10]. Our studies are however the first to demonstrate that factor Xa enhances PDGF-B mRNA and PDGF-BB production and that thrombin also stimulates PDGF-A mRNA and PDGF-AA production by RPE. TGF-β_1 stimulates ECM production and myofibroblastic differentiation and increased levels are present in epiretinal PVR membranes [15, 40]. We found no significant effect of thrombin on TGF-β_1 mRNA expression nor TGF-β_1 secretion by RPE after 24 hours of stimulation, which is in line with previous observations [10]. Production of BMP7, a suppressor of TGF-β_1 signaling [41], was however increased by thrombin. Factor Xa also did not affect

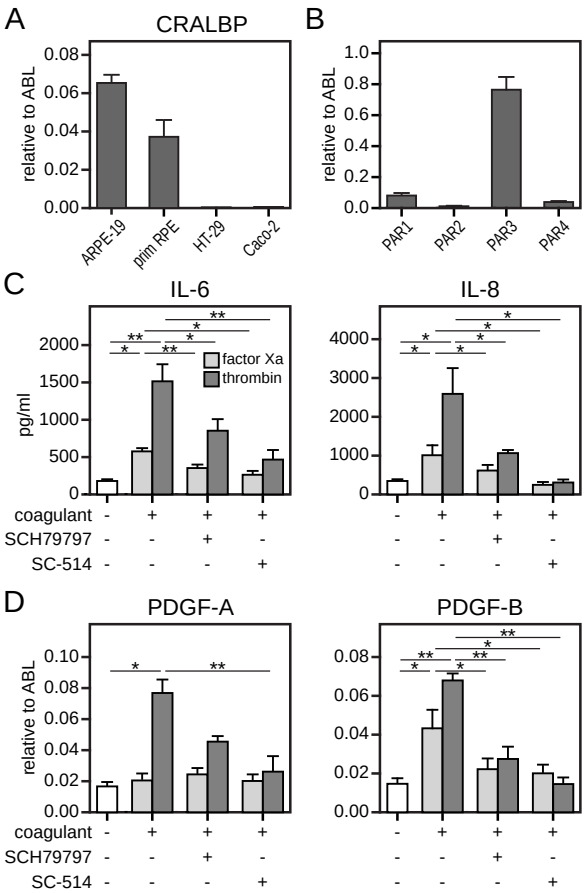

Figure 6: (A) CRALBP mRNA expression was determined on ARPE-19, primary RPE, HT-29 and Caco-2 cells by RQ-PCR and normalized against the control gene ABL. Data are presented as the mean value of 6 independent experiments ± SEM (B) PAR1, PAR2, PAR3 and PAR4 mRNA expression was determined in primary RPE cells by RQ-PCR and normalized against the control gene ABL. Data are presented as the mean value of 5 independent experiments ± SEM. (C) Primary RPE cells were stimulated for 24 hours with factor Xa (1 U/ml) or thrombin (5 U/ml) in the absence or presence of the PAR1 antagonist SCH79797 (250 nM) or the NF-κB inhibitor SC-514 (100 μM). IL-6 and IL-8 concentrations in culture supernatants were determined by ELISA. Data are presented as the mean value of 5 independent experiments ± SEM. (D) Primary RPE cells were stimulated for 4 hours with factor Xa (1 U/ml) or thrombin (5 U/ml) in the absence or presence of the PAR1 antagonist SCH79797 (250 nM) or the NF-κB inhibitor SC-514 (100 μM). PDGF-A and PDGF-B mRNA expression levels were determined by RQ-PCR and normalized against the control gene ABL. Data are presented as the mean value of 5 independent experiments ± SEM. Statistical analysis was performed with the paired Student's t-test. $P < 0.05$ was considered significant. * = $P < 0.05$ and ** = $P < 0.01$.

TGF-β_1 production, but did stimulate the production of TGF-α. TGF-α shares considerable homology with epidermal growth factor (EGF) and binds the EGF-receptor [42]. A role for both TGF-α and EGF-R signaling has been implicated in the pathogenesis of fibrosis [43, 44]. IGF-I is implicated in the pathogenesis of fibrosis, and IGF-I and IGF-II stimulate processes such as proliferation, migration and contraction by RPE cells [45-47]. IGF activity is controlled by the IGFBP family, which contains six different members [48]. Factor Xa and thrombin modulated the production of IGFBP-2, IGFBP-3 and IGFBP-6 by RPE in opposite directions. IGFBP-2 and IGFBP-3 are present in normal vitreous and neutralize IGF-I and IGF-II induced RPE and Müller cell contraction [49]. Although factor Xa and thrombin did not influence IGF-I production by RPE by itself (Supplemental table 1) they can be expected to modulate IGF activity, RPE cell contractility, proliferation and migration by

affecting IGFBP production. Moreover, certain IGFBP-subtypes can directly stimulate fibrotic processes, including epithelial-mesenchymal transition and ECM production [50]. Our data demonstrate that factor Xa and thrombin induce the production of pro-fibrotic mediators by RPE and may thus contribute to fibrotic processes involved in proliferative vitreoretinal disorders.

The blocking experiments with specific PAR antagonists revealed that the effects of factor Xa and thrombin on inflammatory and pro-fibrotic mediator production by RPE were mediated by PAR1. NF-κB inhibition also abrogated the effects of factor Xa and thrombin on inflammatory and pro-fibrotic mediator production by RPE, indicating that factor Xa and thrombin exert their effect via PAR1 mediated activation of the NF-κB signaling pathway.

Thrombin generally stimulated the production of pro-inflammatory cytokines/chemokines and growth factors by RPE more strongly than factor Xa. We regard it unlikely that this is related to the higher thrombin concentration used as our initial titration experiments revealed no further significant increase in IL-6 or IL-8 production when RPE were stimulated with higher concentrations of factor Xa (2 U/ml) (Figure 1). Moreover, thrombin has a much higher affinity for PAR1 than factor Xa [51]. Our data therefore strongly suggest that thrombin more potently stimulates inflammatory and fibrotic responses by RPE than factor Xa.

In conclusion, this study demonstrates that both factor Xa and thrombin activate RPE cells to produce cytokines/chemokines and growth factors that can attract and activate immune cells and induce fibrotic responses. Factor Xa and thrombin may thus contribute to retinal inflammation and fibrosis in vitreoretinal diseases like exudative AMD, PDR and PVR. Given the availability of clinically applicable anti-coagulants, these findings may form the basis of clinical studies to try and prevent the development of (proliferative) vitreoretinal disorders.

Acknowledgments

The authors thank Sandra de Bruin – Versteeg for her help with the illustrations.

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Supplemental table 1

All cytokines/chemokines and growth factors detectable with the quantitative human cytokine antibody array are given in the first column.

The concentration of these factors detected in culture supernatants form ARPE-19 cells in unstimulated situation (US) or after 24 hour stimulation with factor Xa (1 U/ml) or thrombin (5 U/ml) is indicated in the second, third and fourth columns, respectively. The fourth and fifth columns indicate the lower and upper limits of detection, respectively. Cytokines/chemokines and growth factor concentrations are in (pg/ml).

	US	factor Xa	thrombin	lower detection limit	upper detection limit
6Ckine	121.2	95.5	88.8	42.6	80,000
Axl	29.4	31.7	39.3	3.5	8,000
BTC	26.2	16.7	13.3	10.8	40,000
CCL28	142.7	114.0	99.8	34.0	80,000
CTACK	23.7	21.1	22.2	10.8	100,000
CXCL16	5,572.0	5,000.0	5,812.0	10.6	40,000
ENA-78	33.2	24.1	26.8	7.8	20,000
Eotaxin-3	61.6	38.0	48.7	16.2	40,000
GCP-2	16.4	11.8	8.6	9.2	20,000
GRO	15.5	15.4	26.1	0.6	2,000
HCC-1	0.4	0.6	0.3	2.1	8,000
HCC-4	9.4	10.0	9.5	5.1	20,000
IL-9	338.6	164.0	259.2	66.7	400,000
IL-17F	0.0	1.1	0.0	33.5	200,000
IL-18 BPa	143.0	126.9	124.9	36.6	120,000
IL-28A	31.5	20.0	19.3	7.9	20,000
IL-29	424.4	293.9	295.8	90.4	200,000
IL-31	48.4	46.1	44.3	18.8	80,000
IP-10	18.6	12.0	14.8	5.4	20,000
I-TAC	25.2	25.1	14.5	4.3	20,000
LIF	86.0	70.3	107.1	9.4	26,000
LIGHT	35.5	21.7	21.6	9.9	20,000
Lymphotactin	7.6	7.6	3.7	8.8	200,000
MCP-2	5.2	4.6	1.5	1.3	4,000
MCP-3	309.5	376.6	972.8	1.6	8,000
MCP-4	0.9	1.7	0.5	2.5	20,000

	US	factor Xa	thrombin	lower detection limit	upper detection limit
MDC	23.5	23.6	13.3	8.8	20,000
MIF	8,728.9	7,310.8	8,134.5	3.8	8,000
MIP-3α	15.9	15.1	17.8	3.2	8,000
MIP-3β	75.5	49.8	47.7	14.8	40,000
MPIF-1	15.0	13.8	15.0	4.1	20,000
MSPα	350.8	274.6	272.6	44.6	200,000
NAP-2	8.9	5.4	1.4	3.2	8,000
OPN	10,713.2	3,017.4	478.5	58.0	200,000
PARC	19.2	12.4	13.1	2.5	8,000
PF4	3.3	1.7	1.9	6.8	200,000
SDF-1α	10.6	6.3	2.4	4.9	20,000
TARC	18.2	10.1	9.5	3.2	20,000
TECK	170.8	120.8	42.4	111.4	200,000
TSLP	2.6	0.9	0.3	5.2	20,000
BLC	3.3	4.5	2.0	2.1	4,000
Eotaxin	8.9	10.0	8.6	2.6	8,000
Eotaxin-2	1.9	1.9	1.4	1.1	2,000
G-CSF	3.9	7.1	10.6	11.8	40,000
GM-CSF	36.3	91.6	345.0	0.6	2,000
I-309	6.2	4.1	4.5	4.8	8,000
ICAM-1	685.8	973.4	1296.3	22.2	200,000
IFN-γ	6.9	7.4	6.6	2.1	4,000
IL-1α	5.5	5.3	5.8	2.1	4,000
IL-1β	0.7	1.3	0.5	0.6	2,000
IL-1Rα	11.2	14.9	11.8	0.7	4,000
IL-2	9.7	11.3	12.0	1.8	4,000
IL-4	2.4	2.7	4.3	1.3	4,000
IL-5	10.6	12.8	11.0	3.6	8,000
IL-6	2864.4	4596.9	8387.7	2.4	4,000
IL-6sR	1.2	3.1	1.4	3.4	20,000
IL-7	12.4	12.2	11.8	5.2	8,000
IL-8	1482.7	1941.8	3057.8	0.5	1,000
IL-10	2.4	2.6	3.5	2.7	8,000
IL-11	51.2	62.1	98.0	13.0	40,000

	US	factor Xa	thrombin	lower detection limit	upper detection limit
IL-12p40	8.1	9.9	12.8	7.5	20,000
IL-12p70	0.7	0.7	0.9	0.4	1,000
IL-13	1.9	2.1	2.5	0.8	2,000
IL-15	18.7	19.8	22.2	5.3	8,000
IL-16	12.2	13.8	17.8	4.0	10,000
IL-17	34.2	46.4	69.8	3.4	8,000
MCP-1	3632.9	3637.6	6075.6	1.6	4,000
MCSF	10.0	4.1	17.6	2.3	8,000
MIG	24.8	26.6	57.9	1.7	10,000
MIP-1α	11.3	35.3	20.1	4.5	20,000
MIP-1β	0.8	0.0	1.3	0.8	2,000
MIP-1δ	0.1	0.1	0.1	1.4	20,000
PDGF-BB	1.9	7.4	34.5	0.9	4,000
RANTES	0.8	0.9	1.1	5.4	40,000
TIMP-1	10946.6	24790.4	22610.0	4.6	80,000
TIMP-2	32284.1	45967.7	33046.2	11.6	80,000
TNF-α	8.8	16.2	12.3	1.5	4,000
TNF-β	3.0	4.3	2.8	6.4	40,000
TNF RI	966.8	2292.5	3273.2	8.7	80,000
TNF RII	0.0	0.0	0.0	3.7	80,000
AR	30.6	22.6	35.8	9.8	20,000
BDNF	16.4	29.9	25.5	2.3	4,000
bFGF	141.7	119.9	167.8	22.4	40,000
BMP-4	40.4	39.3	25.1	33.5	200,000
BMP-5	333.8	316.6	498.2	94.6	200,000
BMP-7	123.2	163.5	258.2	28.5	80,000
b-NGF	3.9	5.2	7.2	3.4	20,000
EGF	0.4	0.6	0.3	0.2	400
EGF R	913.8	1146.9	800.3	6.3	20,000
EG-VEGF	1.8	2.2	2.1	2.1	20,000
FGF-4	362.3	438.0	241.3	84.9	200,000
FGF-7	3.1	1.8	4.5	5.5	20,000
GDF-15	0.4	2.6	0.8	0.8	4,000
GDNF	9.3	10.2	3.2	4.8	8,000
GH	17.3	21.5	8.0	7.0	20,000

	US	factor Xa	thrombin	lower detection limit	upper detection limit
HB-EGF	15.0	14.1	12.3	6.0	20,000
HGF	4.8	8.7	2.8	3.7	8,000
IGFBP-1	57.4	71.2	69.8	5.4	10,000
IGFBP-2	81.7	57.9	140.8	14.7	40,000
IGFBP-3	324907.4	101131.9	419231.5	156.8	400,000
IGFBP-4	142.3	259.4	88.6	70.4	400,000
IGFBP-6	57717.9	66115.3	32503.4	41.3	200,000
IGF-I	25.8	30.5	35.1	6.5	40,000
Insulin	90.0	94.3	2.7	7.2	40,000
MCF R	5.2	27.4	7.6	21.0	80,000
NGF R	4.5	14.0	2.6	6.3	20,000
NT-3	64.1	43.4	22.6	32.0	80,000
NT-4	3.5	3.5	7.9	2.0	20,000
OPG	1926.9	3220.8	5675.7	2.3	8,000
PDGF-AA	658.4	414.5	1051.6	4.6	20,000
PIGF	0.6	3.4	0.3	2.8	8,000
SCF	0.0	0.2	0.1	1.9	20,000
SCF R	2.5	11.8	5.5	11.2	40,000
TGF-α	612.7	1728.0	716.6	1.1	20,000
TGF-β_1	155.3	222.6	186.6	85.7	200,000
TGF-β_3	8.4	10.3	4.1	24.7	80,000
VEGF	12550.2	12544.0	17015.9	6.9	20,000
VEGF R2	35.0	50.4	33.3	8.1	20,000
VEGF R3	7.9	60.8	3.6	25.8	80,000
VEGF-D	18.0	18.3	20.6	4.7	40,000

Chapter 3

Thrombin induces epithelial-mesenchymal transition and collagen production by retinal pigment epithelial cells via autocrine PDGF-receptor signaling

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Invest Ophthalmol Vis Sci. 2013

Abstract

Purpose: De-differentiation of retinal pigment epithelial (RPE) cells into mesenchymal cells (epithelial-mesenchymal transition; EMT) and associated collagen production contributes to development of proliferative vitreoretinopathy (PVR). In patients with PVR, intraocular coagulation cascade activation occurs and may play an important initiating role. Therefore we examined the effect of the coagulation proteins factor Xa and thrombin on EMT and collagen production by RPE cells.

Methods: RPE cells were stimulated with factor Xa or thrombin and the effect on zonula occludens (ZO)-1, α-smooth muscle actin (α-SMA), collagen and platelet-derived growth factor (PDGF)-B were determined by RQ-PCR, immunofluorescence microscopy and HPLC and ELISA for collagen and PDGF-BB in culture supernatants, respectively. PDGF-receptor activation was determined by phosphorylation analysis and inhibition studies using the PDGF-receptor tyrosine kinase inhibitor AG1296.

Results: Thrombin reduced ZO-1 gene expression ($P < 0.05$) and enhanced expression of the genes encoding α-SMA and the pro-alpha1 chain of collagen type-1 ($P < 0.05$), indicating EMT. Also ZO-1 protein expression declined upon thrombin stimulation while production of α-SMA and collagen increased. In contrast to thrombin, factor Xa hardly stimulated EMT by RPE. Thrombin clearly induced PDGF-BB production and PDGF-Rβ chain phosphorylation in RPE. Moreover AG1296 significantly blocked the effect of thrombin on EMT and collagen production.

Conclusion: Our findings demonstrate that thrombin is a potent inducer of EMT by RPE via autocrine activation of PDGF-receptor signaling. Coagulation cascade-induced EMT of RPE may thus contribute to the formation of fibrotic retinal membranes in PVR and should be considered as treatment target in PVR.

Introduction

Proliferative vitreoretinopathy (PVR) is a complication that is characterized by the formation of fibroproliferative membranes in the retina and develops in approximately 10% of patients that undergo surgery for rhegmatogenous retinal detachment. The fibroproliferative membranes, which primarily contain differentiated retinal pigment epithelial (RPE) cells, fibroblasts, glial cells, immune cells and extracellular matrix (ECM), have contractile properties which causes re-detachment of the retina [1-5].

Epithelial-mesenchymal transition (EMT) is a de-differentiation process in which epithelial cells lose typical epithelial features and acquire mesenchymal features that promote migratory capacity, invasiveness, elevated resistance to apoptosis and strongly enhanced capacity to produce ECM components [5, 6]. EMT is for instance associated with reduced expression of epithelial proteins, such as the tight junction proteins (e.g. zonula occludens (ZO)-1) and adherent junction proteins (e.g. E-cadherin) and enrichment of mesenchymal proteins like α-smooth muscle actin (α-SMA), fibronectin and collagen [6, 7]. EMT or EMT-like processes have been implicated in the development of organ fibrosis, for instance in lungs, liver and kidney, but also the lens of the eye [6, 8]. It has been recognized that RPE cells undergo EMT in PVR membranes and as such are major contributors to the excessive ECM deposition in these membranes [4, 9-11]. Moreover, the associated upregulation of α-SMA contributes to membrane contractility [2, 4]. Although these data point at an important role for EMT by RPE cells in the fibrotic process of PVR there is only limited data available on factors that drive EMT by human RPE. It has been demonstrated that platelet-derived growth factor (PDGF) induces α-SMA expression by human RPE and that PDGF-receptor inhibition prevents EMT and PVR development in experimental animal models [12-15]. A better insight into the process of EMT by human RPE is not only crucial to delineate PVR pathology but is also required to improve treatment strategies, especially since satisfying treatment to prevent PVR development is currently lacking.

Tissue damage results in activation of the extrinsic coagulation pathway with tissue factor dependent activation of factor X to factor Xa. Factor Xa is subsequently involved in the conversion of pro-thrombin into thrombin, which in turn converts soluble fibrinogen into insoluble fibrin [16]. In addition to their role in coagulation, factor Xa and thrombin influence several cellular responses that have important roles in inflammation, tissue repair and wound healing, but that also contribute to fibrogenesis. They can for instance activate endothelial cells, promote chemotaxis of inflammatory cells, stimulate proliferation of fibroblasts, smooth muscle cells and epithelial cells and induce the production of ECM components such as collagen [16-21]. These cellular effects of factor Xa and thrombin are mostly mediated via activation of the cell surface expressed protease activated receptor (PAR)1 and PAR2 [16].

PVR development is associated with breakdown of the blood-retinal barrier and activation of coagulation as evidenced by the deposition of fibrin in the retina and vitreous in PVR patients [22-24]. In line with this, Ricker *et al* demonstrated that subretinal fluids from patients with rhegmatogenous retinal detachment exhibited high capacity to generate thrombin activity in a tissue factor dependent manner [25]. Taken together, this suggests that RPE cells are likely to encounter direct contact with coagulation proteins during PVR development. Previous studies by us and others have demonstrated that factor Xa and thrombin can stimulate the production of cytokines/chemokines, pro-fibrotic and pro-

angiogenic factors by human RPE via PAR1 activation [26-30]. Moreover, there are indications that thrombin induces EMT by rat RPE through transcriptional repression of the epithelial marker E-cadherin and induction of actin stress fibre formation [31, 32]. However, so far it is unknown whether factor Xa and thrombin can induce EMT and collagen production by human RPE.

In this study we examined the effects of factor Xa and thrombin on EMT by the human RPE cell line ARPE-19, as well as in primary human RPE cells. We demonstrate that thrombin stimulates EMT by RPE, as reflected by reduced expression of ZO-1 and enhanced α-SMA expression and collagen production. Furthermore we demonstrate that thrombin stimulates platelet-derived growth factor receptor (PDGF-R)β activity in RPE, most likely via autocrine release of PDGF-BB, and that inhibition of this reverses the effects of thrombin on ZO-1, α-SMA and collagen production. Our data indicate that thrombin is a strong inducer of EMT by RPE via activation of PDGF-receptor signaling.

Materials and methods

Cell cultures

The human retinal pigment epithelial cell line ARPE-19 was obtained from ATCC (Manassas, VA, US). ARPE-19 cells between passages 23-30 were used for experiments. ARPE-19 and primary RPE cells were cultured in RPE medium (DMEM/HAM's F-12 1:1 medium (HyClone Logan, UT, US), containing 10% heat inactivated fetal calf serum (FCS) and penicillin/streptomycin (all from BioWhittaker, Verviers, Belgium)) [26]. The cells were maintained under standard cell culture conditions at 37 °C in humidified air with 5% CO2.

Real-time quantitative PCR analysis

ARPE-19 cells were seeded into six-well plates at a density of 5 x 10^5 cells/well in RPE medium containing 1% FCS and allowed to adhere overnight. Subsequently the cells were incubated for 0, 12, 24, 36 or 48 hours in the presence or absence of human factor Xa (1 U/ml) or thrombin (5 U/ml) (Calbiochem, La Jolla, CA, US) (the concentrations used are based on findings in a previous study [26]) in RPE medium containing 1% FCS for analysis of *ACTA2* (encoding for α-SMA) and *COL1A1* (encoding for the pro-alpha1 chain of type I collagen) transcript levels. For analysis of *TJP1* (encoding for ZO-1) transcript levels ARPE-19 cells were seeded into six-well plates at a density of 5 x 10^5 cells/well in RPE medium containing 1% FCS. Cells were allowed to adhere overnight followed by an additional 7-9 days in order to enable tight junction formation, while refreshing medium containing 1 % FCS every 3-4 days. Subsequently the cells were incubated for 0, 12, 24, 36 or 48 hours in the presence or absence of human factor Xa (1 U/ml) or thrombin (5 U/ml) in RPE medium containing 1% FCS. RNA was isolated using a GenElute™ Mammalian Total RNA Miniprep Kit (Sigma; St. Louis, MO, US) and reverse transcribed into cDNA [33]. Transcript levels of *ACTA2*, *COL1A1*, and *TJP1* were determined by RQ-PCR (7700 PCR system; Applied Biosystems [ABI], Foster City, CA, US). *PDGFRA* (PDGF-Rα chain) and *PDGFRB* (PDGF-Rβ chain) mRNA expression levels were determined in unstimulated ARPE-19 cells. In addition, ARPE-19 cells were stimulated with factor Xa (1 U/ml) or thrombin (5 U/ml) for 0, 1, 2, 4, 6 or 8 hours and the effect on *PDGFB* mRNA expression level was determined. Expression levels of the analyzed gene transcripts were normalized to the control gene *ABL* (Abelson) [33]. Primer and probe combinations used are listed in table 1.

Fluorescent immunocytochemistry

For analysis of α-SMA protein expression, ARPE-19 cells were cultured on 12 mm cover slips for 2-3 days in RPE medium containing 0.5% FCS. The cells were stimulated with factor Xa (1 U/ml), thrombin (5 U/ml) or remained unstimulated for an additional 48 hours. Thereafter, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 10 min. After washing twice with PBS, fixed cells were incubated with glycine (300 mM) for 5 minutes and then permeabilized with PBS containing 0.1% Triton X-100 for 10 min. Cells were then washed twice with PBS containing 0.1% Tween 20, incubated with PBS containing 1% fatty acid free BSA and 0.1% Tween 20 for 1 hour and then incubated overnight with rabbit anti-human-α-SMA primary antibody (clone E184, 04-1094; Millipore, Billerica, MD, US) at 4 °C. Thereafter, cells were washed three times with PBS containing 0.1% Tween 20 and subsequently incubated for 1 hour with Alexa Fluor594-conjugated chicken anti-rabbit secondary antibody (Invitrogen, Paisley, UK) in PBS containing 1% BSA, 0.1% Tween 20 and 10% human serum. Following secondary antibody

incubation, cells were washed six times with PBS containing 0.1% Tween 20 and then incubated with the nuclear stain DAPI (Invitrogen) for 10 min. Cells were analyzed by fluorescence microscopy.
For analysis of ZO-1 protein expression, ARPE-19 cells were cultured on 12 mm cover slips for 7-9 days in RPE medium containing 1% FCS to allow formation of tight junctions. Stimulation and staining of the cells were conducted as described above, using overnight incubation with Alexa Fluor488-conjugated mouse-anti-human ZO-1 primary antibody (clone ZO-1-1A12, 339188; Invitrogen) at 4 °C, followed by DAPI staining.

For analysis of collagen type 1 expression, ARPE-19 cells were cultured on 12 mm cover slips for 2-3 days in RPE medium containing 0.5% FCS. Twenty-four hours prior to stimulation and during the 48 hour stimulation period 50 µg/ml ascorbic acid and 200 µM L-Proline (Sigma) was added to the culture medium. After the first 24 hours again 50 µg/ml ascorbic acid and 200 µM L-Proline was added to the cell cultures. Here the cells were incubated overnight with a mouse-anti-human collagen type 1 primary antibody (clone 5D8-G9, MAB3391; Millipore) at 4 °C, 1 hour incubation with FITC-conjugated rabbit anti-mouse secondary antibody (Dako, Heverlee, Belgium) and finally stained with DAPI.

Collagen measurement in culture supernatants
ARPE-19 cells were seeded into six-well plates at a density of 1 x 10^5 cells/well in RPE medium containing 1% FCS and allowed to grow till 100% confluence. Twenty-four hours prior to stimulation 50 µg/ml ascorbic acid and 200 µM L-proline was added to the culture media. Subsequently the cells were incubated in fresh RPE medium containing 1% FCS, 50 µg/ml ascorbic acid and 200 µM L-proline for 24 or 48 hours in the presence or absence of factor Xa (1 U/ml) or thrombin (5 U/ml). In case of 48 hour incubation 50 µg/ml ascorbic acid and 200 µM L-proline was again added to the cell cultures after the first 24 hours of incubation. Following stimulation, the cells and medium were harvested together and proteins were ethanol-precipitated in 67% ethanol [21]. Collagen was assessed by measuring hydroxyproline levels in proteins by high-pressure liquid chromatography, as previously described [21]. The amount of collagen present in the culture supernatants was calculated based on the fact that collagen contains 12.2% w/w hydroxyproline and expressed relative to the amount produced by unstimulated cells [34].

Detection of PDGF receptors and PDGF receptor phosphorylation
In order to determine protein expression levels of the PDGF-Rα and PDGF-Rβ chains in ARPE-19 cells, unstimulated cells were lysed in protein lysis buffer (20 mM Tris pH 8.0, 137 mM NaCl, 10 mM EDTA, 100 mM NaF, 1% NP-40, 10% glycerol and protease inhibitors). Equal amounts of protein (25 µg) were loaded on SDS-PAGE and blotted onto a nitrocellulose membrane. Western blots were stained with rabbit-anti human PDGF-Rα (clone C-20, SC-338), rabbit-anti human PDGF-Rβ (clone P-20, SC-339) (Santa Cruz Biotechnologies, Heidelberg, Germany) or mouse-anti human β-actin (clone AC-15, AB6276; Abcam, Cambridge, UK) antibodies. In order to assess PDGF-receptor phosphorylation, cultures were stimulated with factor Xa (1 U/ml), thrombin (5 U/ml), PDGF-BB (50 ng/ml) (R&D Systems, Minneapolis, MN, US) as positive control, or remained unstimulated for 8 hours in RPE medium containing 1% FCS. Hereafter cells were lysed in protein lysis buffer and cell lysates (~500 µg) were used for immunoprecipitation using an anti-PDGF-R

antibody as described [35]. Immune complexes were collected with a mixture of protein A- and G-Sepharose (Sigma) and the level of PDGF-Rβ phosphorylation was determined by using an anti-phospho-tyrosine antibody (#9411; Cell Signaling, Danvers, MA, US) as described [35] and quantified by densitometric analysis (ImageQuant TL; GE Healthcare, Freiburg, Germany).

PDGF-BB detection in culture supernatants
ARPE-19 cells were seeded into six-well plates at a density of 5 x 10^5 cells/well in RPE medium containing 1% FCS and allowed to adhere overnight. Subsequently the cells were incubated for 24 hours in the presence or absence of human factor Xa (1 U/ml) or thrombin (5 U/ml) in RPE medium containing 1% FCS. Supernatants were harvested and PDGF-BB levels were measured using a PDGF-BB ELISA (R&D Systems) according manufacturer's instructions.

PDGF receptor inhibition studies
RPE cells were seeded into six-well plates at a density of 5 x 10^5 cells/well in RPE medium containing 1% FCS and allowed to adhere overnight. Hereafter the cells were incubated with RPE medium containing 10 μM of the PDGF-receptor tyrosine kinase inhibitor AG1296 (Calbiochem) for 60 minutes and subsequently stimulated for 12 hours with factor Xa (1 U/ml), thrombin (5 U/ml) or PDGF-BB (50 ng/ml) as positive control. In order to determine the effect of AG1296 on *TJP1* mRNA expression the seeded cells were cultured for 7-9 days in RPE medium containing 1% FCS. Hereafter the cells were incubated with fresh RPE medium containing 10 μM AG1296 for 60 minutes and subsequently stimulated for 24 hours with factor Xa (1 U/ml), thrombin (5 U/ml) or PDGF-BB (50 ng/ml). The effect of AG1296 on *ACTA2*, *TJP1* and *COL1A1* mRNA expression was determined by RQ-PCR. The AG1296 concentration used was non-toxic to RPE cells as determined by lactate dehydrogenase (LDH) release (Roche, Mannheim, Germany) and microscopic appearance of the cells.

Statistical analysis
Data were analysed using the Kruskal-Wallis (One-way ANOVA) test followed by the paired Student's *t*-test when applicable. A *P-value* < 0.05 was considered significant.

Results

The effect of factor Xa and thrombin on α-SMA and ZO-1 expression by RPE

Factor Xa and thrombin enhanced *ACTA2* mRNA expression by ARPE-19, with maximal levels at 12 hours following stimulation and with thrombin significantly ($P < 0.05$) being more potent (Figure 1A). In line with this, 48 hours stimulation with thrombin clearly enhanced the expression of α-SMA filaments in ARPE-19, while this was less evident for factor Xa (Figure 1B). *TJP1* mRNA expression was significantly ($P < 0.05$) reduced 24 hours after thrombin stimulation and was still reduced at 48 hours. Factor Xa also appeared to decrease *TJP1* mRNA expression, but this effect did not reach significance (Figure 1C). Forty-eight hours stimulation with thrombin also clearly reduced ZO-1 protein expression by ARPE-19, while this was not observed with factor Xa (Figure 1D). Taken together, these results show that thrombin strongly enhances α-SMA expression and reduces ZO-1 expression by ARPE-19 cells while the expression of these proteins is hardly influenced by factor Xa.

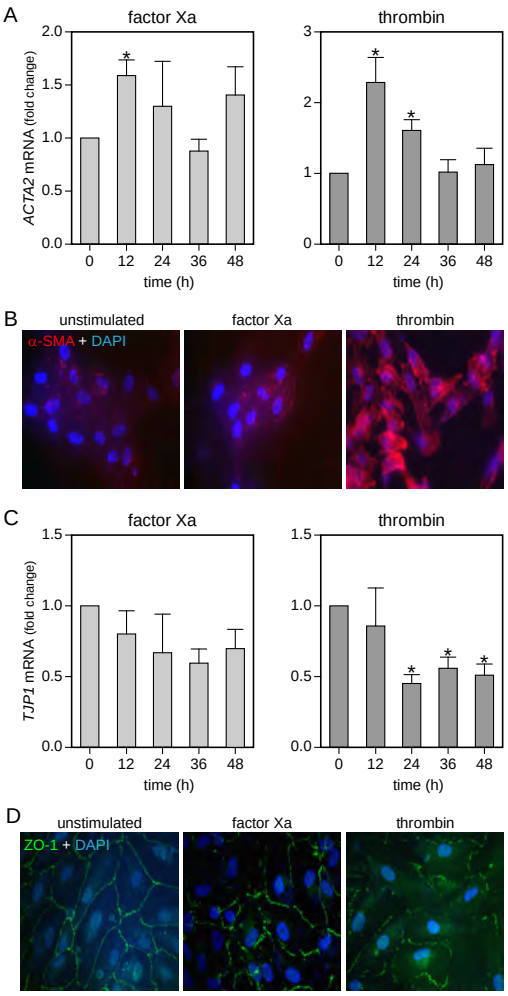

Figure 1: (A) ARPE-19 cells were stimulated for 0, 12, 24, 36 and 48 hours with factor Xa (1 U/ml) or thrombin (5 U/ml) and the ACTA2 mRNA expression level was determined by RQ-PCR and normalized against the control gene ABL. (B) ARPE-19 cells were stimulated for 48 hours with factor Xa (1 U/ml) or thrombin (5 U/ml) and the cells were fixed and stained with anti-alpha smooth muscle actin (primary antibody (rabbit anti-human) and Alexa Fluor594-conjugated secondary antibody (chicken anti-rabbit)). The nuclei were stained with DAPI. (C) ARPE-19 cells were stimulated for 0, 12, 24, 36 and 48 hours with factor Xa (1 U/ml) or thrombin (5 U/ml) and the TJP1 mRNA expression level was determined by RQ-PCR and normalized against the control gene ABL. (D) ARPE-19 cells were stimulated for 48 hours with factor Xa (1 U/ml) or thrombin (5 U/ml) and the cells were fixed and stained with Alexa Fluor488-conjugated anti-ZO-1 antibody (mouse anti-human). The nuclei were stained with DAPI. RQ-PCR data are presented as the mean value from 4 independent experiments ± SEM. Statistical analysis was performed with the Kruskal-Wallis (One-way ANOVA) test followed by the paired Student's t-test when applicable. $P < 0.05$ was considered significant. * = $P < 0.05$ compared to t = 0h. Immunofluorescence pictures are representative for 3 independent experiments.

The effect of factor Xa and thrombin on collagen production by RPE

Factor Xa and thrombin stimulation resulted in significantly ($P < 0.05$ and $P < 0.01$, respectively) elevated levels of *COL1A1* mRNA following 12 hours after stimulation. For thrombin this elevation was sustained up to at least 36 hours following stimulation and returned to base-line level 48 hours following stimulation (Figure 2A). Moreover, collagen type 1 protein expression was clearly enhanced after 48 hours of thrombin stimulation, while this was not the case for factor Xa (Figure 2B). These findings were confirmed by the collagen levels detected in the culture supernatants, demonstrating a significant ($P < 0.05$) increase in collagen secretion by RPE after 48 hours of thrombin stimulation (Figure 2C). Collectively this data demonstrates that thrombin strongly enhances collagen production by RPE cells.

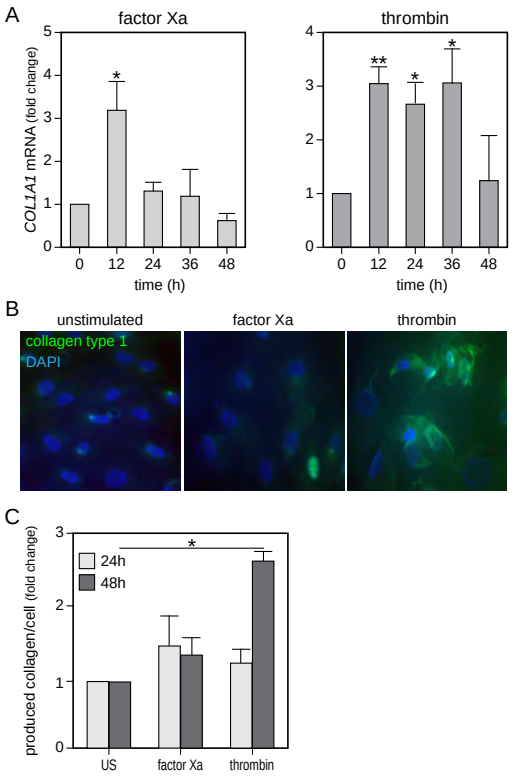

Figure 2: (A) ARPE-19 cells were stimulated for 0, 12, 24, 36 and 48 hours with factor Xa (1U/ml) or thrombin (5 U/ml) and the COL1A1 mRNA expression level was determined by RQ-PCR and normalized against the control gene ABL. (B) ARPE-19 cells were stimulated for 48 hours with factor Xa (1 U/ml) or thrombin (5 U/ml) and the cells were fixed and stained with anti-collagen type 1 primary antibody (mouse anti-human) and FITC-conjugated secondary antibody (rabbit anti-mouse). The nuclei were stained with DAPI. (C) ARPE-19 cells were stimulated for 24 or 48 hours with factor Xa (1 U/ml) or thrombin (5 U/ml) and after stimulation the cells numbers were determined and hydroxyproline content in the culture supernatants was determined by HPLC. RQ-PCR and HPLC data are presented as the mean value from 4-5 independent experiments ± SEM. Statistical analysis was performed with the Kruskal-Wallis (One-way ANOVA) test followed by the paired Student's t-test when applicable. $P < 0.05$ was considered significant. * = $P < 0.05$ and ** = $P < 0.01$ compared to t = 0h or unstimulated (US). Immunofluorescence pictures are representative for 3 independent experiments.

The effect of factor Xa and thrombin on PDGF receptor phosphorylation

PDGF-isoforms can stimulate α-SMA expression and collagen production by various cell types, including RPE [13, 36, 37]. Previously we found that factor Xa but much stronger thrombin stimulated the production of PDGF-AA, PDGF-AB and PDGF-BB by RPE cells (ARPE-19 and primary RPE), and here we identified thrombin as a potent inducer of EMT by RPE [26]. Collectively, these data suggest that thrombin may exert its EMT effect

in RPE in an indirect manner that involves activation of PDGF-receptor signaling. PDGF molecules signal via homodimeric or heterodimeric receptors consisting of PDGF-Rα and/or PDGF-Rβ chains [38]. Therefore we first examined PDGF-receptor chain expression in ARPE-19. Analysis of mRNA and protein expression of the PDGF-Rα and PDGF-Rβ chains revealed that ARPE-19 cells abundantly express PDGF-Rβ chains, but hardly express PDGF-Rα chains (Figure 3A). Similar results were found in primary RPE (data not shown). This suggests that PDGF signaling in ARPE-19 will most likely predominantly occur via PDGF-Rβ homodimers. PDGF-BB can signal via PDGF-Rβ-homodimers, while PDGF-AB and AA signal via PDGF-Rα containing dimers [38]. As expected stimulation of ARPE-19 with PDGF-BB resulted in significant ($P < 0.05$) phosphorylation of PDGF-Rβ chains, indicating receptor activation (Figure 3B). In ARPE-19 cells, factor Xa hardly stimulated PDGF-Rβ chain phosphorylation, while thrombin significantly ($P < 0.05$) stimulated resulted PDGF-Rβ chain phosphorylation (Figure 3B).

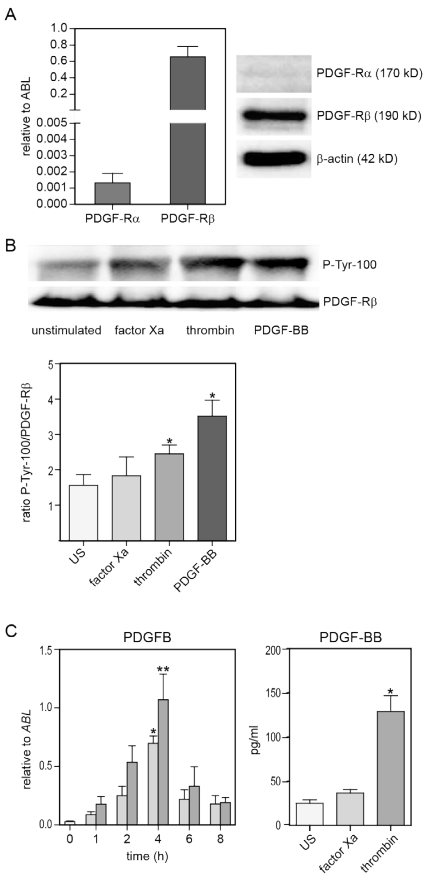

Figure 3: (A) PDGFRA and PDGFRB mRNA expression levels in unstimulated ARPE-19 cells were determined by RQ-PCR and normalized against the control gene ABL. Data are presented as the mean value of 4 independent experiments ± SEM. PDGF-Rα, PDGF-Rβ and β-actin protein expression levels in unstimulated ARPE-19 cells were determined by Western Blot. (B) ARPE-19 cells were stimulated for 8 hours with or without factor Xa (1 U/ml), thrombin (5 U/ml) or PDGF-BB (50 ng/ml). Total lysates from these stimulated cells were used for immunoprecipitation using an anti-PDGF-Rβ antibody. Immune complexes were collected with a mixture of protein A- and G-Sepharose and the PDGF-Rβ levels were determined. Levels of PDGF-Rβ phosphorylation were determined by using an anti-phospho-tyrosine antibody. The depicted western blot is representative for 3 independent experiments. Densitometric analysis was performed and the P-Tyr-100/PDGF-Rβ ratio was determined. Data are presented as the mean value from 3 independent experiments ± SEM. (C) ARPE-19 cells were stimulated for 0, 1, 2, 4, 6 and 8 hours with factor Xa (1U/ml) or thrombin (5 U/ml) and the PDGFB mRNA expression levels were determined by RQ-PCR and normalized against the control gene ABL. Data are presented as the mean value from 4 independent experiments ± SEM. ARPE-19 cells were stimulated for 24 hours with or without factor Xa (1U/ml) or thrombin (5 U/ml) and PDGF-BB protein expression levels were determined. Data are presented as the mean value from 4 independent experiments ± SEM. Statistical analysis was performed with the Kruskal-Wallis (One-way ANOVA) test followed by the paired Student's t-test when applicable. $P < 0.05$ was considered significant. * = $P < 0.05$ and ** = $P < 0.01$ compared to t = 0h or US. US = unstimulated.

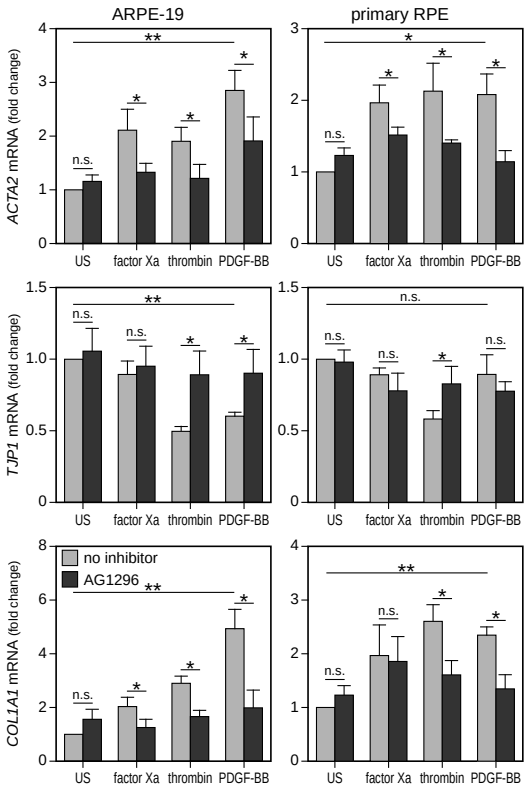

Figure 4: ARPE-19 cells and primary RPE were stimulated for 12 (ACTA2 and COL1A1) or 24 hours (TJP1) with factor Xa (1 U/ml), thrombin (5 U/ml) or PDGF-BB (50 ng/ml) in the absence or presence of the inhibitor AG1296 (10 µM). ACTA2, TJP1 and COL1A1 mRNA expression levels were determined by RQ-PCR and normalized against the control gene ABL. Data are presented as the mean value from 5-6 independent experiments ± SEM. Statistical analysis was performed with the Kruskal-Wallis (One-way ANOVA) test followed by the paired Student's t-test when applicable. $P < 0.05$ was considered significant. * = $P < 0.05$ and ** = $P < 0.01$ compared to t = 0h. US = unstimulated.

Table 1: PCR primer and probe sequences

Gene	Sequence fwd primer (5`-3`)
ABL [26]	TGGAGATAACATCTAAGCATAACTAAAGGT
ACTA2	CCGACCGAATGCAGAAGGA
COL1A1	ACTGGCCCCCCTGGTCC
PDGFB [26]	TCCCGAGGAGCTTTATGAGATG
PDGFRA [58]	TGAAGGCAGGCACATTTACATCTA
PDGFRB [58]	GGGGACAGGGAGGTGGATT
TJP1	GAGACGCTGGAACTGACCAA

The effect of factor Xa and thrombin on PDGF-BB production

Next we examined whether the lower level of PDGF-Rβ phosphorylation induced by factor Xa as compared to thrombin related to kinetic differences in PDGF-BB induction. Hereto, ARPE-19 cells were stimulated with factor Xa and thrombin for different time points and the effect on *PDGFB* mRNA expression was determined. This revealed that factor Xa and thrombin enhanced *PDGFB* mRNA expression in a time depending manner, being maximal and significant (thrombin $P < 0.01$, factor Xa $P < 0.05$) 4 hours after stimulation with thrombin being the most potent (Figure 3C). In line with this, 24 hour thrombin stimulation resulted in a significant ($P < 0.05$) increase in PDGF-BB secretion by ARPE-19 cells, while the increase induced by factor Xa was not significant (Figure 3C). These data combined demonstrate that thrombin is the more potent inducer of PDGF-BB expression.

The effect of PDGF-receptor inhibition on the regulation of α-SMA, ZO-1 and collagen type 1 expression by factor Xa and thrombin

In order to examine whether the effect of thrombin on α-SMA, ZO-1 and collagen type 1 expression by RPE depends on PDGF-receptor activation we performed inhibition studies with the PDGF-receptor tyrosine kinase inhibitor AG1296, which potently inhibits signaling of human PDGF-Rα and –Rβ. PDGF-BB significantly ($P < 0.05$) enhanced *ACTA2* and *COL1A1* mRNA expression by both ARPE-19 and primary RPE. PDGF-BB significantly ($P < 0.05$) reduced *TJP1* mRNA levels in the ARPE-19 cells, but not in the primary RPE cells. The effects of PDGF-BB on *ACTA2*, *TJP1* and *COL1A1* mRNA levels were completely reversed ($P < 0.05$) by AG1296 (Figure 4). In line with previous results (Figures 1 and 2) thrombin significantly ($P < 0.05$) enhanced *ACTA2* and *COL1A1* mRNA expression and reduced *TJP1* mRNA expression by ARPE-19. Comparable effects for thrombin on *ACTA2*, *COL1A1* and *TJP1* mRNA levels were observed in primary RPE (Figure 4). These effects of thrombin were significantly ($P < 0.05$) blocked by AG1296. Also the factor Xa stimulated increase in *ACTA2* and *COL1A1* mRNA levels in ARPE-19 and primary RPE cells was blocked by AG1296.

	Sequence rev primer (5`-3`)	Sequence probe (5`-3`)
	GATGTAGTTGCTTGGGACCCA	FAM-CCATTTTTGGTTTGGGCTTCACACCATT-TAMRA
	ACAGAGTATTTGCGCTCCGGA	FAM-ATCACGGCCCTAGCACCCAGCA-TAMRA
	GGGCTCTCCAGCAGCACCTT	FAM-CCGGACCCCCAGGCCCACCT-TAMRA
	CGGGTCATGTTCAGGTCCAAC	FAM-AGTGACCACTCGATCCGCTCCTTTG-TAMRA
	TACAGGAGTCTCGGGATCAGTTG	FAM-TGCCAGACCCAGATGTAGCCTTTGTACCTC-TAMRA
	ATTCCCGATCACAATGCACA	FAM-TCTACAGACTCCAGGTGTCATCCATCAACGTC-TAMRA
	TGTTTGTCTTGATCTATGATTTGCTT	FAM-AGCTCTGGCATTATTCGCCTGCATACA-TAMRA

Discussion

EMT contributes to fibrotic processes, including those occurring in the retina of patients with PVR [2, 13, 39, 40]. Blood-retinal barrier breakdown and associated activation of the coagulation cascade contributes to PVR development [25, 41]. Here we demonstrate that the coagulation protein thrombin is a strong inducer of EMT by human RPE and that factor Xa does this to a far lesser extent.

EMT occurs in response to injury to promote wound closure and tissue regeneration. However in fibrosis, which can be regarded as a dysregulated healing attempt, EMT programs are not attenuated, thereby contributing to excessive matrix production [42]. We found that thrombin downregulated the expression of epithelial tight junction protein ZO-1 while expression of the contractile protein α-SMA was enhanced. This transformation of RPE cells into contractile myofibroblast like cells allows migration into the vitreous where they contribute to PVR membrane formation and the contractile properties of these membranes [5, 7]. Myofibroblast like cells appear to be the main cell type responsible for collagen accumulation in fibrosis [43, 44]. In line with this, we found that thrombin-induced downregulation of ZO-1 and upregulation of α-SMA was accompanied by enhanced production of collagen, which is one of the main constituents of PVR membranes [45]. The effects of factor Xa on EMT and collagen production were less pronounced. We regard this unlikely to be related to the concentration used as we previously observed that 1U/ml factor Xa generated maximal PAR1 activation in RPE [26]. Activation of RPE by thrombin may thus represent a major route of coagulation factor-driven EMT in PVR.

Fibrin clots have been suggested to contribute to fibrosis in general by providing ECM producing cells with a scaffold for adherence, proliferation and ECM production [22, 28]. Along this hypothesis, retinal fibrin deposition has been suggested to promote RPE cell proliferation, differentiation and collagen synthesis in PVR [25, 41]. However, experiments in fibrinogen knock-out mice revealed that bleomycin-induced lung fibrosis was not diminished, while direct blockade of PAR1 did inhibit bleomycin-induced lung fibrosis in mice [46, 47]. These data indicate that fibrin deposition is not per se required for fibrosis development and that especially the cell mediated effects of thrombin and factor Xa contribute to fibrosis development in this model. Here we demonstrate that especially thrombin induces EMT and collagen production by human RPE, while others have shown that thrombin stimulates human RPE cell migration and rat RPE cell proliferation [28, 48]. Moreover, we and others found that factor Xa and thrombin stimulate the production of several cytokines and growth factors by RPE via PAR1 activation [26-30]. Therefore the direct receptor-mediated cellular effects that thrombin and factor Xa elicit in RPE by activation of PAR1 may play an important role in PVR development.

PDGF-receptor activity has been observed in human PVR membranes and PDGF has been found to stimulate EMT by RPE [10, 13, 49, 50]. PDGF-receptor chains dimerize into functional signaling units upon ligand binding [38]. Here we found that ARPE-19 as well as primary RPE predominantly express the PDGF-Rβ chain, which is in line with previous observations [51]. Consequently RPE cells can be expected to predominantly form PDGF-Rβ homodimers, which is activated by PDGF-BB [38]. In our current study we confirm our previous finding that thrombin induces PDGF-BB production by RPE more potent than factor Xa [26]. In line with this we here demonstrate that thrombin clearly enhances PDGF-Rβ chain phosphorylation (activation) in ARPE-19, while factor Xa does this to a far lesser extent.

Moreover, the effects of factor Xa and thrombin on *ACTA2*, *TJP1* and *COL1A1* mRNA expression were blocked by the PDGF-receptor tyrosine kinase inhibitor AG1296. Thus thrombin, and to a lesser extent factor Xa, most likely induce EMT by RPE via PDGF-Rβ signaling initiated via autocrine release of PDGF-BB. Despite of low expression of PDGF-Rα by RPE cells, activation of this receptor is considered important to PVR development [50, 52]. We can not exclude involvement of PDGF-Rα in our study as it can be activated by PDGF-A and PDGF-B chain containing PDGF-dimers that are induced by factor Xa and thrombin in RPE (this study), while AG1296 also blocks PDGF-Rα activation [26, 38]. Transactivation of PDGF- Rα via other growth factor receptors, for instance the FGF- or EGF- receptor can also not be excluded [53, 54]. Although, we consider this unlikely since we previously demonstrated that thrombin nor factor Xa stimulate FGF and EGF production by RPE [26]. Nevertheless, our data clearly indicate that that the stimulatory effects of thrombin on EMT of RPE involves PDGF-Rβ activation.

Factor Xa had a far less pronounced effect on EMT, collagen production and PDGF-Rβ phosphorylation by RPE than thrombin in our studies. Whether this relates to the fact that factor Xa requires cofactors such as tissue factor, endothelial protein C receptor or annexin 2 for efficient PAR1 cleavage and a relative absence of such factors in our culture system is unclear [55-57]. However, we found that factor Xa stimulates PDGF-BB secretion by ARPE-19 to a lesser extent than thrombin and that factor Xa less potently but with comparable kinetics to thrombin enhanced *PDGFB* mRNA expression in ARPE-19. This indicates that production of a certain amount of PDGF and subsequent level of PDGF-receptor activation has to be achieved by the coagulation factor to efficiently induce EMT by RPE.

Collectively, our previous and current findings demonstrate that coagulation cascade activity can induce EMT of RPE via autocrine PDGF-receptor signaling (Figure 5) [26]. Inhibition of thrombin activity or its activator factor Xa by using clinically applicable anti-coagulants may therefore be considered in the treatment of PVR. Alternatively targeting of the downstream PDGF-receptor signaling cascade with specific PDGF-receptor targeting tyrosine kinase inhibitors or the use of PAR1 antagonists may also be of therapeutic interest.

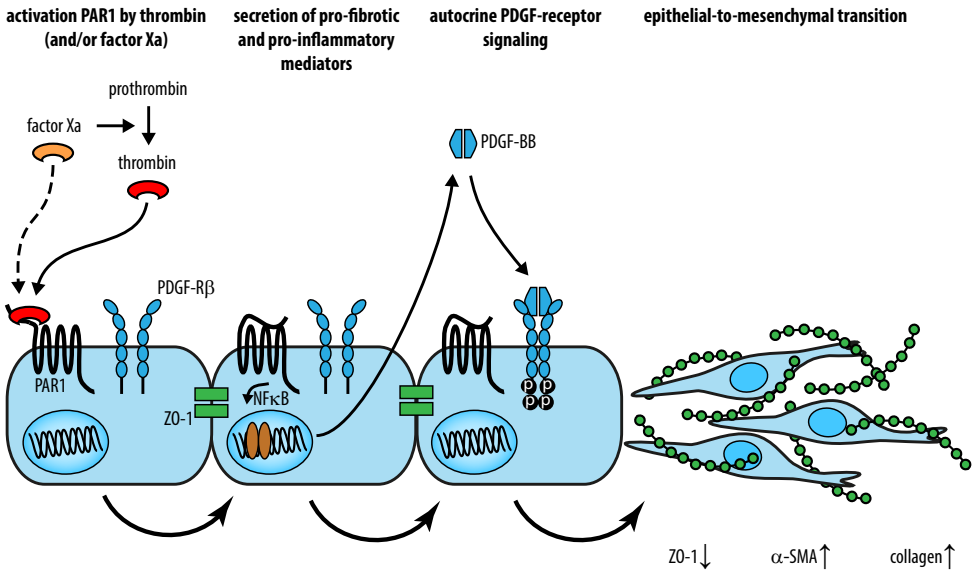

Figure 5: Schematic summary of the activation of RPE by factor Xa and thrombin resulting in EMT. Factor Xa and thrombin activate PAR1 which results in NF-κB signaling and the secretion of a broad panel of pro-inflammatory cytokines and growth factors, including PDGF-BB [26]. The secreted PDGF-BB binds an PDGF-Rβ chain resulting in the formation of PDGF-Rβ dimers, autophosphorylation and activation of PDGF-receptor signaling. Signaling through the PDGF-receptor results in differentiation of RPE into a mesenchymal cell type characterized by decreased ZO-1 expression and increased α-SMA expression and collagen production. The dashed line between factor Xa and PAR1 indicates a weaker effect compared to thrombin.

Acknowledgments

The authors would like to thank dr. Sharada Karanam from the Centre for Inflammation and Tissue Repair, University College London for her technical support and Sandra de Bruin – Versteeg from the Dept. of Immunology, Erasmus MC for her help with the illustrations.

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Chapter 4

Monocyte-macrophage differentiation is induced in thrombin-stimulated monocyte-ARPE-19 co-cultures

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Manuscript in preparation

Abstract

Background: Macrophages play an important role in proliferative vitreoretinopathy (PVR) and may therefore represent an attractive therapeutic target. Macrophage differentiation is modulated by the local environment, but how this is controlled in PVR is largely unknown. Besides macrophages retinal pigment epithelial (RPE) cells are also important contributors to PVR development. Recently it was found that PVR is associated with increased intra-ocular thrombin activity. In this study we determined the effect of thrombin on RPE cells interacting with monocytes on macrophage differentiation.

Methods: Human monocytes and the human RPE cell line ARPE-19 were cultured separately or in co-culture in the presence or absence of thrombin. Macrophage differentiation was examined by immunocytochemistry for CD68. Experiments using neutralizing antibodies against integrin β2 (CD18) and ICAM-1 (CD54) were performed to study the contribution of these adhesion molecules. CXCL8, ICAM-1, IL-6, IL-10, PDGF-BB, TGF-β_1, TGF-β_2 and VEGF-A levels were measured in culture supernatants with ELISA.

Results: Thrombin strongly enhanced monocyte-to-macrophage differentiation in monocyte-ARPE-19 co-cultures. This was abrogated by neutralizing antibodies against CD18 or CD54. Macrophage differentiation in the presence of ARPE-19 and thrombin was associated with significantly ($P < 0.05$) higher production of soluble ICAM-1, PDGF-BB and VEGF-A.

Conclusions: Thrombin supports monocyte-to-macrophage differentiation when co-cultured with ARPE-19, which involves the adhesion molecules CD18 and CD54. This macrophage differentiation is associated with elevated secretion of the growth factors PDGF-BB and VEGF-A, suggestive for M2-maturation. Thrombin activity together with activated RPE and monocytes support the maturation of macrophages. This process may contribute to the development of PVR.

Introduction

Proliferative vitreoretinopathy (PVR) is a fibrotic disorder of the retina that can develop after rhegmatogenous retinal detachment and is the most common failure of retinal detachment surgery [1]. PVR development is characterized by the formation of subretinal, and/or epiretinal fibroproliferative membranes and intraretinal fibrosis, with excessive amounts of extracellular matrix (ECM) molecules and contractile myofibroblasts that cause the retina to detach [2, 3]. PVR leads to visual impairment and can result in complete loss of vision if not treated in time. However, besides (repeated) surgery there is currently no satisfying medical treatment available for the treatment or prevention of PVR, likely because the pathobiology of PVR is still poorly understood.

The fibroproliferative contractile membranes represent the final pathological end-stage of PVR and are formed through a complex interplay between different cell types, specific receptors and soluble molecules [4-10]. Retinal pigment epithelial (RPE) cells are considered key players in the formation of these membranes, since they exhibit the capacity to de-differentiate into myofibroblasts (epithelial mesenchymal transition; EMT) that are potent producers of ECM molecules [1, 11].

In case of disturbed tissue homeostasis monocytes are recruited from the peripheral blood into tissue where they differentiate into macrophages [12]. Macrophages are specialized phagocytes that remove tissue debris and microbial components but they also respond to diverse environmental signals that drive further differentiation [13, 14]. When macrophages adopt the alternatively activated M2-phenotype, a process driven by factors including granulocyte macrophage colony stimulating factor (GM-CSF), interleukin (IL)-4 and IL-13, they can hugely contribute to fibrosis [15, 16]. M2-macrophages secrete pro-fibrotic growth factors including platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF) [17]. These growth factors support proliferation of different cell types, including fibroblasts and epithelial cells, stimulate EMT and promote ECM molecule production as well as stabilization of already deposited ECM components [18-23]. PDGF, TGF-β and VEGF, are all elevated in vitreous of patients with established PVR [1-3, 24, 25]. In addition, cytokines/chemokines and adhesion molecules including chemokine (C-C motif) ligand (CCL)2, CCL3, CCL4, chemokine (C-X-C motif) ligand (CXCL)10 and intercellular adhesion molecule (ICAM)-1, that recruit monocytes and stimulate subsequent macrophage differentiation, are elevated in PVR vitreous [26-28]. In line with this, monocytes and macrophages are abundantly present in PVR membranes and vitreous [1, 24, 26, 28-30]. Therefore, macrophages are considered as important cellular source of growth factors that stimulate pro-fibrotic events, including EMT and ECM production by RPE cells, in PVR. Macrophages may thus represent a cellular target to prevent PVR development. However, the mechanisms involved in macrophage differentiation in PVR are hardly examined so far.

PVR is associated with increased intravitreal activity of the coagulation protease thrombin that stimulates the production of CCL2, CCL7 and GM-CSF by RPE cells, which are all chemoattractants for monocytes while GM-CSF also stimulates the differentiation of monocytes into macrophages [11, 31-34]. In addition, it has been described that thrombin stimulates ICAM-1 expression by RPE cells and strengthens the physical interaction between RPE cells and monocytes when co-cultured [32, 33].

The data above suggest that thrombin activated RPE cells may facilitate retinal macrophage accumulation in PVR and may thus represent an attractive pharmacological

target to interfere with macrophage activity. In the current study we demonstrate for the first time that thrombin-activated monocyte-RPE co-cultures induce macrophage differentiation associated with increased production of the pro-fibrotic mediators PDGF-BB and VEGF-A and to a lesser extent TGF-β_1 and TGF-β_2. This suggests that under these conditions thrombin drives the generation of pro-fibrotic M2-type macrophages. Moreover this differentiation process likely depends on physical interaction between monocytes and RPE cells facilitated by adhesion molecules including ICAM-1 (CD54) and β2-integrin (CD18).

Materials and methods

Monocyte isolation

Ficoll (Ficoll-PaqueTM Plus, GE Healthcare Bio-Sciences AB, Uppsala) density gradient separation was used to obtain peripheral blood mononuclear cells (PBMC) from buffy coat samples (Sanquin, Amsterdam, The Netherlands). Monocytes were subsequently isolated from the PBMC by using CD14-beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and a autoMACS Pro Seperator (Miltenyi Biotec GmbH) according to the manufactures' instructions. Isolated monocytes were cultured in RPMI1640 culture medium containing 10% heat-inactivated fetal calf serum (FCS) and 1% penicillin/streptomycin (all from BioWhittaker, Verviers, Belgium) for 24 hours.

ARPE-19 culture

The human retinal pigment epithelial cell line ARPE-19 was obtained from ATCC (Manassas, VA, US). ARPE-19 were cultured in RPE medium (DMEM/F-12 medium; HyClone, Logan, UT, US), containing 10% FCS and penicillin/streptomycin (all from BioWhittaker). For experiments ARPE-19 cells between passage 23-27 were used. The cells were maintained under standard cell culture conditions at 37 °C in humidified air with 5% CO_2.

Immunocytochemical analysis

ARPE-19 were seeded on cover slips (24x24 mm Menzel-Gläser; Portsmouth, New Hampshire, US), which were placed inside the wells of 6-well plates (Nunc; Roskilde, Denmark), at a density of 5 x 10^5 cells/well in RPE medium containing 10% FCS and allowed to adhere overnight. Subsequently the medium was refreshed twice a week till the cells were grown 100% confluent. Prior to stimulation, both ARPE-19 and monocytes were serum starved in RPMI1640 medium containing 1% FCS. Monocytes, 5 x 10^5 cells/well, and a monolayer of ARPE-19 cells, separately or in co-culture were pre-incubated with fresh RPMI1640 medium with or without 7.5 U/ml of the thrombin inhibitor hirudin (Sigma Aldrich, St. Louis, MO, US) for 1 hour and subsequently stimulated with 5 U/ml thrombin (Calbiochem, La Jolla, CA, US) in the presence or absence of 7.5 U/ml hirudin for an additional 72 hours. Hereafter the culture medium and non-adherent cells were removed. Cells remaining on the glass slides were washed twice with PBS (pH 7.4) followed by fixation with 4% paraformaldehyde for 10 minutes where after the slides were again washed twice with PBS followed by permeabilization with PBS containing 0.1% Triton X-100 for 4 minutes. Slides were then washed twice with PBS containing 0.05% Tween20 and incubated with PBS containing 10% normal rabbit serum and 1% bovine serum albumin (BSA) for 10 minutes, followed by a single wash step in PBS containing 0.05% Tween20. Subsequently mouse anti-human CD68 (1/100; clone EBM11; Dako, Heverlee, Belgium) in PBS containing 1% BSA was applied for 1 hour. Thereafter, cells were washed twice with PBS containing 0.05% Tween20 and incubated for 30 minutes with rabbit anti-mouse Ig (1/400; Dako) in PBS containing 1% BSA and thereafter washed twice with PBS containing 0.05% Tween20. Then streptavidin Poly-HRP conjugate (Thermo Fisher Scientific, Waltham, MA, US) in PBS containing 1% BSA was added for 1 hour followed by two washes with PBS and subsequent incubation with aminoethyl carbazole (AEC) solution for 10 minutes. Finally the cells on the slides were washed twice with PBS and stained with hematoxylin and eosin. All steps were performed at room temperature. After rinsing the cells with tap water the cells on the cover slip were covered with Kaiser's Glycerol/Gela-

tin (Boom BV; Meppel, The Netherlands) and placed upside down on microscope slides (Menzel). The slides were analyzed by microscopy and photographs were made using Axiocam (Zeiss, Sliedrecht, The Netherlands).

Neutralization experiments

ARPE-19 were seeded on cover slips, which were placed inside the wells of 6-well plates, at a density of 5 x 10^5 cells/well in RPE medium containing 10% FCS and allowed to adhere overnight. Subsequently the medium was refreshed twice a week till the cells were grown 100% confluent. Prior to stimulation, both ARPE-19 and monocytes were serum starved in RPMI1640 medium containing 1% FCS. Monocytes, 5 x 10^5 cells/well and a monolayer of ARPE-19 cells in co-culture were pre-incubated with fresh RPMI1640 medium with or without 1 µg/ml mouse anti-human CD18 (clone MEM-148; Abcam, Cambridge, UK), 1µg/ml mouse anti-human CD54 (clone P2A4; Millipore, Billerica, MD, US) or 1 µg/ml mouse-IgG1 (isotype control, clone 11711; R&D systems) for 1 hour. Subsequently the cells were stimulated for 72 hours in RPMI1640 medium containing 1% FCS with or without 5 U/ml thrombin and with or without 1 µg/ml mouse anti-human CD18, 1 µg/ml mouse anti-human CD54 or 1 µg/ml mouse-IgG1. Following the stimulation period of 72 hours culture supernatants were removed and cells on the glass slides were stained for CD68 positive cells as described above.

Detection of cytokines, chemokines and growth factors

ARPE-19 were seeded into 6-well plates at a density of 5 x 10^5 cells/well in RPE medium containing 10% FCS and allowed to adhere overnight. Subsequently the medium was refreshed twice a week till the cells were grown 100% confluent. Prior to stimulation, both ARPE-19 and monocytes were serum starved in RPMI1640 medium containing 1% FCS. Monocytes, 5 x 10^5 cells/well, and a monolayer of ARPE-19 cells separately or in co-culture were stimulated with 5 U/ml thrombin for 72 hours. Following the stimulation period culture supernatants were collected and analyzed by ELISA for the M1 associated mediators CXCL8 and IL-6 (Invitrogen; Paisley, UK) and the M2 associated mediators IL-10, PDGF-BB, TGF-β_1, TGF-β_2 and VEGF-A (R&D Systems; Abingdon, UK) and ICAM-1 (R&D Systems) according manufacturer's instructions.

Statistical analysis

ELISA data were analyzed using the Kruskal-Wallis (one-way ANOVA) test followed by the Mann-Whitney *U* test when applicable. A *P* value < 0.05 was considered significant.

Results

The effect of thrombin on ARPE-19-mediated macrophage differentiation

Monocytes, either stimulated with thrombin or unstimulated, did not adhere to the glass slides making further analysis into macrophage differentiation impossible. Co-culture of monocytes with ARPE-19 resulted in the generation of some CD68 positive macrophages (Figure 1). Stimulation of the monocyte-ARPE-19 co-cultures with thrombin resulted in a morphological change of the ARPE-19 cells that became spindle-shaped fibroblast resembling cells (Figure 1). This morphological change by ARPE-19 was also observed when ARPE-19 was stimulated with thrombin in the absence of monocytes and is in line with our previous observation that thrombin stimulates de-differentiation of RPE-cells into myofibroblasts [11]. Moreover, stimulation of the monocyte-ARPE-19 co-cultures with thrombin clearly enhanced the generation of CD68 positive macrophages (Figure 1). Monocytes used in these experiments were CD68 negative prior to stimulation as was measured by flow cytometry (data not shown).

To demonstrate that the effect truly depended on thrombin activity the experiments were repeated in the presence of the direct thrombin inhibitor hirudin. Hirudin clearly reduced the thrombin-induced generation of CD68 macrophages when monocytes were co-cultured with ARPE-19 (Figure 1).

The role of adhesion molecules on thrombin-induced ARPE-19-mediated macrophage differentiation

Addition of neutralizing antibodies against CD18 or CD54 to the thrombin-stimulated monocyte-ARPE-19 co-cultures inhibited the generation of CD68 expressing macrophages while the isotype control did not (Figure 2).

Cytokine, chemokine and growth factor levels in culture supernatants

Monocytes hardly produced any CXCL8, IL-6, ICAM-1, PDGF-BB, TGF-β_1, TGF-β_2 and VEGF-A and neither was this stimulated by thrombin (Figure 3). ARPE-19 produced CXCL8, IL-6, ICAM-1, PDGF-BB, TGF-β_1, TGF-β_2 and VEGF-A under basal culture condition (Figure 3). Thrombin significantly ($P < 0.05$) enhanced CXCL8, IL-6, ICAM-1, PDGF-BB, TGF-β_1 and VEGF-A production by ARPE-19, while it had no statistically significant effect on TGF-β_2 production (Figure 3). Co-culture of monocytes with ARPE-19 resulted in significantly ($P < 0.05$) higher production of ICAM-1 and PDGF-BB compared to the levels produced by ARPE-19 alone (Figure 3). Stimulation of the monocyte-ARPE-19 co-cultures with thrombin resulted in significantly ($P < 0.05$) higher production of ICAM-1, PDGF-BB and VEGF-A compared to unstimulated co-cultures (Figure 3).

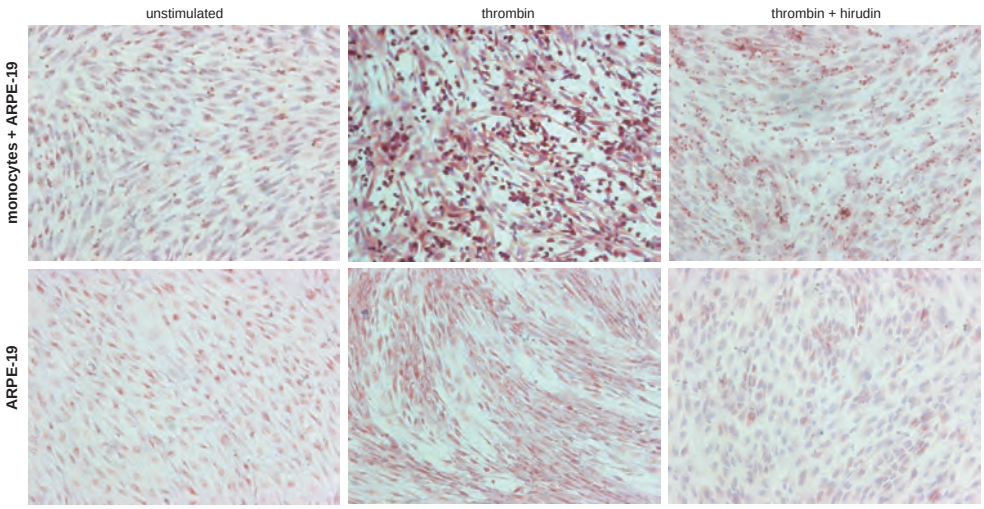

Figure 1: Monocytes and ARPE-19 cells were cultured on glass slides separately and in co-culture for 72 hours. The cells were stimulated with thrombin (5 U/ml) or remained unstimulated in the presence or absence of the direct thrombin inhibitor hirudin (7.5 U/ml). Non-adhered cells were washed away and adhered cells were stained with CD68 to identify macrophages. Representative example is depicted.

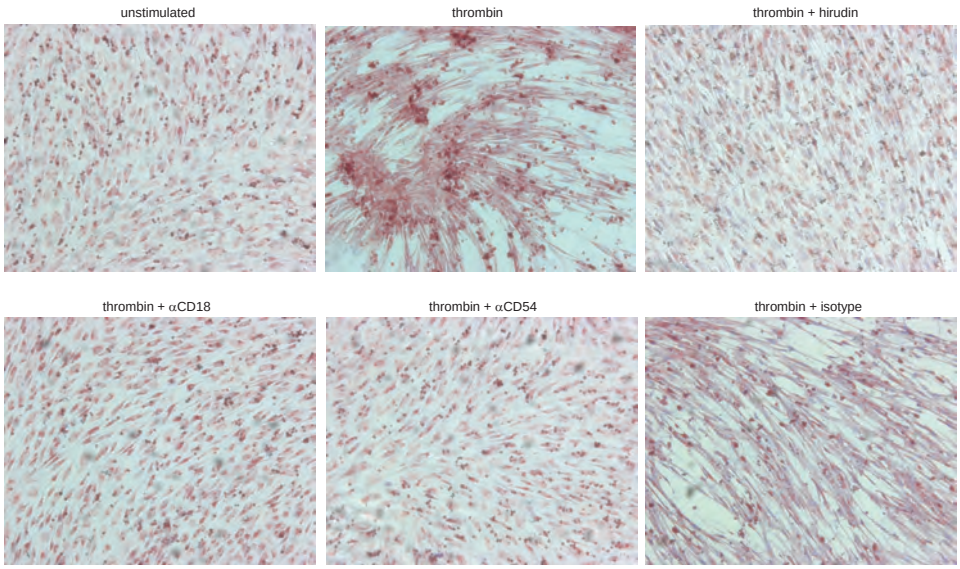

Figure 2: Monocytes and ARPE-19 cells were co-cultured on glass slides for 72 hours. The cells were stimulated with thrombin (5 U/ml) or remained unstimulated in the presence or absence of the direct thrombin-inhibitor hirudin (7.5 U/ml) or neutralizing antibodies against CD18, CD54 or isotype control (each at 1 μg/ml). Non-adhered cells were washed away and adhered cells were stained with CD68 to identify macrophages. Representative example is depicted.

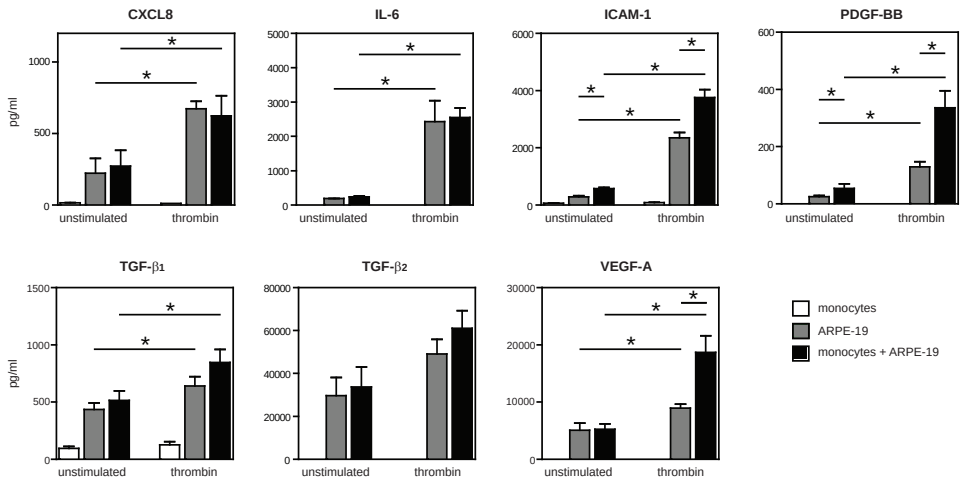

Figure 3: Monocytes and ARPE-19 cells were cultured separately and in co-culture for 72 hours. The cells were stimulated with thrombin (5 U/ml) or remained unstimulated. Culture supernatants were analyzed for CXCL8, IL-6, IL-10, ICAM-1, PDGF-BB, TGF-β_1, TGF-β_2 and VEGF-A by ELISA. Data are presented as the mean value from five independent experiments ± SEM. Statistical analysis was performed with the Kruskal-Wallis (one-way ANOVA) test followed by the Mann-Whitney *U* test when applicable. A *P* value < 0.05 was considered significant. **P* < 0.05.

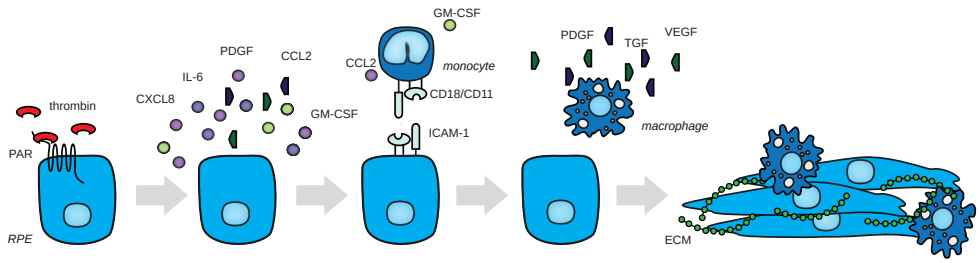

Figure 4: Schematic overview of proposed model: Activation of RPE by thrombin results in increased production of cytokines, chemokines and growth factors. The produced cytokines induce ICAM-1expression by RPE and produced chemokines recruit monocytes. The presence of GM-CSF and cell-cell contact between monocytes and RPE via their adhesion molecules, including at least CD18 and ICAM-1, induce M2-macrophage differentiation. Growth factors produced by both thrombin-activated RPE and newly formed M2-macrophages induce fibrotic processes such as proliferation, migration, EMT and ECM production by at least the RPE cells. All these processes combined result in the formation of fibroproliferative membranes which can cause the retina to detach.

Discussion

Macrophages are important contributors to PVR via the secretion of growth factors that stimulate fibrosis. This would make them interesting cellular targets for therapy, if such agents were available. However, the mechanism of macrophage differentiation in PVR is so far hardly understood. Recently, we described that PVR development is associated with increased intraocular thrombin activity [31]. Here we demonstrate that thrombin strongly enhances macrophage formation from monocytes when co-cultured with the human RPE cell line ARPE-19. Neutralizing antibodies against the adhesion molecules β2-integrin (CD18) or ICAM-1 (CD54) abrogate this macrophage differentiation. Moreover, macrophage differentiation in this thrombin-monocyte-ARPE-19 co-culture model is associated with high production of the pro-fibrotic growth factors PDGF-BB and VEGF-A.
In previous studies Osusky *et al* examined interactions between human monocytes and RPE cells. They demonstrated that co-culture of human monocytes with RPE cells resulted in increased proliferation of RPE. This effect was greatly reduced when direct cell contact was prevented when monocytes and RPE were separated by a membrane in a transwell culture system [35]. In other studies this group demonstrated that monocyte-RPE co-culture resulted in monocyte-macrophage differentiation, which involved the adhesion molecule CD11c as well as TGF-β activity [36, 37]. In line with these findings we observed that co-culture of monocytes with ARPE-19 was associated with elevated secretion of PDGF-BB, a potent mitogen for RPE cells [19, 38]. In our study thrombin further enhanced the PDGF-BB production in the monocyte-ARPE-19 cultures, suggesting that this might further enhance the proliferative activity of RPE cells in PVR. Comparable to Osusky *et al*, we observed macrophage differentiation when peripheral blood derived monocytes where co-cultured with ARPE-19. This macrophage differentiation was drastically enhanced when thrombin was present in the monocyte-ARPE-19 co-cultures and was associated with a significantly higher level of soluble ICAM-1.
ICAM-1 interacts with other adhesion molecules, including lymphocyte function-associated antigen-1 (LFA-1: CD11a/CD18), macrophage adhesion ligand-1 (MAC-1: CD11b/CD18) and the integrin p150,95 (CD11c/CD18; type 4 complement receptor), which are expressed by myeloid cells and are required for adhesion and chemotaxis [39]. Here we found that neutralizing antibodies against CD18 or ICAM-1 abrogated the monocyte-to-macrophage differentiation when co-cultured with ARPE-19 and thrombin. ICAM-1 is absent from resting leukocytes but moderately expressed on activated leukocytes, including monocytes/macrophages. Moreover, ICAM-1 is expressed on epithelia, including RPE, which is enhanced upon activation with inflammatory stimuli [40]. CD18 is expressed at high level by especially leukocytes although human RPE cells might express CD18 as well [41]. Although we did not examine CD18 and CD54 expression levels under the culture conditions studied we expect that ICAM-1 was mostly expressed by ARPE-19 and CD18 by the monocyte/macrophages. Nevertheless the data so far suggest that physical interaction between monocytes and ARPE-19, mediated by at least CD18 and ICAM-1, is involved in the enhanced monocyte-to-macrophage differentiation when thrombin is present (Figure 4). We can however not exclude that soluble factors, including soluble ICAM-1 but also GM-CSF that are induced in RPE by thrombin, contributed as well [32]. Experiments using transwell culture systems will provide more insight into this process (work in progress).

Macrophages can adopt an M1 or M2-phenotype that can be distinguished by expression of specific surface molecules and signature cytokines and growth factors [15, 17, 42]. M1 macrophages are pro-inflammatory via secretion of for instance tumor necrosis factor (TNF)-α and IL-6 [43, 44]. M2-macrophages are anti-inflammatory and contribute to healing/tissue remodeling responses through secretion of high levels of mediators like IL-10, TGF-β, PDGF and VEGF [42]. In this study PDGF-BB and VEGF-A levels were highly elevated in the thrombin-monocyte-ARPE-19 co-culture supernatants (Figure 3). Moreover, there was a trend toward higher levels of TGF-β_1, TGF-β_2 in thrombin-monocyte-ARPE-19 co-culture supernatants compared to thrombin-stimulated ARPE-19 monoculture supernatants (Figure 3). We did so far not examine expression of M1/M2 related surface molecules (e.g. CD163 for M1 and CD206 for M2 [45]) but our results demonstrate that monocytes co-cultured with ARPE-19 in the presence of thrombin differentiated into macrophages, which was associated with enhanced production of M2-related growth factors (esp. PDGF-BB and VEGF-A) and not M1-related cytokines (CXCL8 and IL-6). A more extensive analysis of both cellular and soluble markers (e.g. interleukin-1 receptor antagonist, pentraxin-related protein, TNF-α) might generate better insight into the subtype of macrophage generated (work in progress) [43, 44]. However, so far we assume that the increased levels of PDGF-BB and VEGF-A from the thrombin-stimulated co-cultures are derived from M2 differentiated macrophages. It can however not be excluded that ARPE-19 contributed to the elevated levels as well.

Based on our current findings and our previous observation that PVR is associated with increased intraocular thrombin activity we propose the model depicted in Figure 4 where thrombin facilitates the interaction between monocytes and RPE cells and subsequent differentiation of pro-fibrotic M2-macrophages [31]. These findings indicate that thrombin inhibition may reduce the development of pro-fibrotic M2-macrophages in those patients at risk for PVR.

Acknowledgements

The authors would like to thank Marjan van Meurs, Nada Ristya Rahmani and Rosanne Veerman for their technical support. The research for this study was (in part) performed within the framework of the Erasmus Postgraduate School Molecular Medicine.

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Chapter 5

The role of thrombin in proliferative vitreoretinopathy

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Invest Ophthalmol Vis Sci. 2014 Jul

Abstract

Purpose: To determine the role of thrombin in the development of proliferative vitreoretinopathy.

Methods: Vitreous was collected from patients undergoing a vitrectomy (macular holes and puckers n = 11 (controls), retinal detachment without PVR development following vitrectomy n = 15 (RRD1), retinal detachment with PVR development within 6 months after vitrectomy n = 11 (RRD2), and established PVR n = 14 (PVR)). Thrombin activity in vitreous was determined using a thrombin-specific chromogenic substrate. ARPE-19 cells were stimulated with 8 times diluted vitreous samples in the presence and absence of hirudin. The samples were analyzed at t = 0 and t = 24 hours for the presence of 27 cytokines/chemokines and growth factors using a multiplex approach. In comparable studies, ARPE-19 cells were stimulated for 2 hours and mRNA expression levels for *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* were determined by RQ-PCR.

Results: Thrombin activity was significantly ($P < 0.05$) higher in vitreous of the PVR group compared to the other groups. PVR vitreous stimulated the production CCL2, CXCL8, GM-CSF, IL-6 and PDGF-BB by ARPE-19 to significantly ($P < 0.05$) higher levels that vitreous from the RRD1 and RRD2 groups. These effects of PVR vitreous were significantly ($P < 0.05$) reduced by hirudin. These data were confirmed by mRNA studies.

Conclusion: Thrombin activity is increased in vitreous of patients with established PVR and is involved in the activation of pro-inflammatory and pro-fibrotic pathways in RPE cells. Inhibition of thrombin activity may therefore represent a potential treatment option for proliferative vitreoretinopathy.

Introduction

Proliferative vitreoretinopathy (PVR) is an inflammatory fibrotic disorder which can develop after rhegmatogenous retinal detachment, and is the most common failure of retinal detachment repair. PVR development is characterized by the formation of subretinal, intraretinal and/or epiretinal fibroproliferative membranes that cause the retina to detach due to the contractile properties of myofibroblasts that are abundantly present in these membranes [1, 2]. Retinal pigment epithelial (RPE) cells contribute to the formation of these fibroproliferative membranes through the secretion of cytokines and growth factors, proliferation, and de-differentiation into extracellular matrix producing myofibroblasts [1, 3, 4]. However, our current knowledge of the underlying pathobiological processes in PVR is still limited, which may explain why no clear improvement in medical therapy has been achieved during the last decades. PVR development is associated with blood-retinal barrier breakdown and activation of the coagulation system, as evidenced by fibrin deposition in retina and vitreous from PVR patients [5-7]. Thrombin is the terminal coagulation enzyme that converts soluble fibrinogen into the insoluble fibrin clot, and subretinal fluids from retinal detachment patients have been described to contain high capacity to generate thrombin activity [6, 8]. Besides its central role in coagulation, thrombin stimulates many cellular processes involved in inflammation, wound repair and fibrosis in a variety of cell types, including RPE cells, by activating the high affinity thrombin receptor: protease-activated receptor (PAR)1 [5, 9-13]. Recently we demonstrated that thrombin stimulates human RPE cells to produce a wide variety of cytokines, chemokines and growth factors, amongst which chemokine (C-C motif) ligand (CCL)7, chemokine (C-X-C motif) ligand (CXCL)8, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6, platelet-derived growth factor (PDGF)-AA and PDGF-BB are associated with PVR development [9, 10, 14-17]. Although these data suggest that thrombin can contribute to PVR development, no data are present on thrombin activity in vitreous samples and its contribution to RPE cell activation in PVR.

Here we examined intravitreal thrombin activity in PVR. This study is the first to demonstrate that vitreous from PVR patients contains increased thrombin activity which contributes to vitreous-induced production of cytokines/chemokines and growth factors by RPE cells. Our data indicate that elevated intravitreal thrombin activity contributes to PVR development, at least by inducing pro-inflammatory and pro-fibrotic responses in RPE cells. Intravitreal thrombin activity may represent a novel biomarker for PVR development, and thrombin inhibition may be considered as therapeutic option in the treatment of PVR.

Methods

Vitreous fluids

Vitreous samples varying from 0.6 - 1.2 ml from the following groups of patients that underwent a standard three-port vitrectomy were used for this study: 1) macular holes n = 3 and idiopathic macular puckers n = 8, which served as controls, 2) rhegmatogenous retinal detachment without PVR development after vitrectomy (RRD1, n = 15), 3) rhegmatogenous retinal detachment with PVR development within 6 months following vitrectomy (RRD2, n = 11), and 4) established PVR (PVR, n = 14). The vitreous samples were collected from 2010-2013, centrifuged at 1500 RPM and stored at -20 °C for a maximum of 7 days before long term storage at -80 °C. All subjects gave their consent for the use of rest material for research; storage and use of the vitreous for further studies was according to the guidelines of the Medical Ethics Committee of the Erasmus MC, University Medical Center Rotterdam. Patient characteristics are given in table 1.

Table 1: Patients and Controls

Group	Description	Sex, Male/ Female	Age, y
Controls	Macular holes and macular puckers	6/5	74.73 ± 6.07
RRD1	Rhegmatogenous retinal detachment (no PVR development; 6 months after vitrectomy)	10/4	57.33 ± 6.89
RRD2	Rhegmatogenous retinal detachment (with PVR development; 6 months after vitrectomy)	11/4	64.64 ± 19.82
PVR	Established PVR	6/5	59.36 ± 11.24

Cell cultures

The human retinal pigment epithelial cell line ARPE-19 was obtained from ATCC (Manassas, VA, US). ARPE-19 cells were cultured in RPE medium (DMEM/HAM's F-12 1:1 medium (HyClone, Logan, UT, US), containing 10% heat inactivated fetal calf serum (FCS) and penicillin/streptomycin (all from BioWhittaker, Verviers, Belgium)) and between passage 23-30 when used for experiments. The cells were maintained under standard cell culture conditions at 37 °C in humidified air with 5% CO_2.

Thrombin activity measurement in vitreous fluids

Thrombin activity in vitreous was determined by using a thrombin-specific substrate as we and others did previously for bronchoalveolar lavage fluid [18, 19]. In short, 25 µl vitreous fluid was diluted in a 96-well microtiter plate with 25 µl Tris-buffered saline (TBS pH 8.3) or TBS containing 0.1 U/ml of the specific thrombin inhibitor hirudin (Sigma, St Louis, MO, US). A thrombin (Calbiochem, La Jolla, CA, US) standard curve ranging from 50 - 0.78 milli units per ml (mU/ml) was prepared in TBS or TBS containing 0.1 U/ml hirudin. All solutions were incubated for 30 minutes at 37 °C to allow formation of thrombin-hirudin complexes. Thereafter, 50 µl of a 1 mM solution of the thrombin specific substrate Tos-Gly-Pro-Arg-pNA (Sigma), dissolved in 1.5 mM HCl, was added to the (diluted) samples and incubated at 37 °C. The optical density (OD) was measured at 405 nm after 8 hours. Thrombin activity in vitreous fluid was quantified based on the difference in OD between vitreous fluid with and without hirudin and the reference curve and expressed as mU/ml.

Determining the effect of different vitreous dilutions on IL6 and PDGFB mRNA expression by ARPE-19

ARPE-19 cells were seeded in 12-wells plates at a density of 3 x 10^5 cells/well in RPE medium containing 10% FCS and allowed to adhere overnight. Subsequently the medium was refreshed twice a week till the cells were grown 100% confluent. Prior to stimulation, the cells were serum starved in RPE medium containing 1% FCS for 24 hours followed by an additional 24 hours in serum free RPE medium. Hereafter, cells were stimulated for 1, 2 or 4 hours with vitreous of 4 PVR patients diluted 1/4 and 1/8 in serum free RPE medium. RNA was isolated using a GenElute™ Mammalian Total RNA Miniprep Kit (Sigma) and reverse transcribed into cDNA [10]. Transcript levels of *IL6* and *PDGFB* mRNA were determined by real-time quantitative (RQ)-PCR (7700 PCR system; Applied Biosystems [ABI], Foster City, CA, US). Expression levels of the analyzed gene transcripts were normalized to the control gene *ABL* (Abelson) [10]. Primer and probe combinations used are listed in table 2.

Measurement of vitreous-induced cytokine, chemokine and growth factor secretion by ARPE-19

ARPE-19 cells were seeded in 12-wells plates at a density of 3 x 10^5 cells/well in RPE medium containing 10% FCS and allowed to adhere overnight. Subsequently the medium was refreshed twice a week till the cells were grown 100% confluent. Prior to stimulation, the cells were serum starved in RPE medium containing 1% FCS for 24 hours followed by an additional 24 hours in serum free RPE medium without FCS. Hereafter cells were pre-incubated with fresh serum free RPE medium with or without 7.5 U/ml hirudin for 60 minutes. Subsequently the cells were stimulated for 24 hours with serum free medium containing 1/8 diluted vitreous (average thrombin concentration per sample for RRD1: 0,699 ± 0,14 mU/ml, RRD2: 0,917 ± 0,38 mU/ml and PVR; 5,678 ± 1,88 mU/ml) or 5 U/ml thrombin with or without 7.5 U/ml hirudin. Following the stimulation period of 24 hours (T24) culture supernatants were harvested for cytokine, chemokine and growth factor analysis. In addition, an aliquot of 125 µl of the prepared 1/8 diluted vitreous samples (t = 0 hours; T0) was stored at -20 °C for determination of basal cytokine, chemokine and

Table 2: Primer and Probe Sequences

Gene	Sequence Forward Primer, 5'–3'
ABL	TGGAGATAACATCTAAGCATAACTAAAGGT
CXCL8	GGCCGTGGCTCTCTTGG
GMCSF	CCGCCTGGAGCTGTACAA
IL6	TAGCCGCCCCACACAGA
PDGFB	TCCCGAGGAGCTTTATGAGATG

growth factor levels. To samples and T24 culture supernatants were analyzed with a Bio-Plex Pro™ Human Cytokine, Chemokine, and Growth Factor Assay (Bio-Rad, Hercules, CA, US) allowing simultaneous detection of the following cytokines, chemokines and growth factors: IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, FGFb, Eotaxin, G-CSF, GM-CSF, IFNγ, IP-10/CXCL10, MCP-1/CCL2, MIP-1α/CCL3, MIP-1β/CCL4, PDGF-BB, RANTES, TNF-α, VEGF-A (see supplemental table 1 for the detection limits). The assay was performed according manufacturer's instructions.

Measurement of PVR vitreous-induced cytokine, chemokine and growth factor mRNA expression levels in ARPE-19

ARPE-19 cells were seeded in 12-wells plates at a density of 3 x 10^5 cells/well in RPE medium containing 10% FCS and allowed to adhere overnight. Subsequently the medium was refreshed twice a week till the cells were grown 100% confluent. Prior to stimulation, the cells were serum starved in RPE medium containing 1% FCS for 24 hours followed by an additional 24 hours in serum free RPE medium. Hereafter, cells were pre-incubated with serum free RPE medium with or without 7.5 U/ml hirudin for 60 minutes and subsequently stimulated for 2 hours with serum free medium containing 1/8 diluted PVR vitreous (average thrombin concentration per sample; 4,211 ± 0,60 mU/ml) with or without 7.5 U/ml hirudin. RNA was isolated and reverse transcribed into cDNA [10]. Transcript levels of *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* were determined by RQ-PCR. Expression levels of the analyzed gene transcripts were normalized to the control gene *ABL* [10]. *CCL2* transcript levels were determined using a commercially available Taqman Gene Expression assay (Applied Biosystems). Other primer and probe combinations used are listed in table 2.

Statistical analysis

Messenger RNA and protein data were analyzed using the Kruskal-Wallis (One-way ANOVA) test followed by the Mann-Whitney U test when applicable. A *P*-value < 0.05 was considered significant.

	Sequence Reverse Primer, 5'–3'	Sequence Probe, 5'–3'
	GATGTAGTTGCTTGGGACCCA	FAM-CCATTTTTGGTTTGGGCTTCACACCATT-TAMRA
	GGGTGGAAAGGTTTGGAGTATGT	FAM-TGTGTGAAGGTGCAGTTTTGCCAAGGA-TAMRA
	AGGGGATGACAAGCAGAAAGTC	FAM-TTGCACAGGAAGTTTCCGGGGTTG-TAMRA
	GTGCCTCTTTGCTGCTTTCAC	FAM-AGCCACTCACCTCTTCAGAACGAATTGACA-TAMRA
	CGGGTCATGTTCAGGTCCAAC	FAM-AGTGACCACTCGATCCGCTCCTTTG-TAMRA

Results

Thrombin activity in vitreous

Thrombin activity was hardly present in the vitreous of patients from the control group (2.48 ±1.25 mU/ml, mean value ± standard error of the mean (SEM), Figure 1). Vitreous samples from patients with rhegmatogenous retinal detachment without PVR development in a later stage (RRD1) displayed slightly, but not significantly, higher thrombin activity (9.93 ± 1.86 mU/ml) than the control group. Thrombin activity in vitreous samples of patients with rhegmatogenous retinal detachment that did develop PVR within 6 months after vitrectomy (RRD2) showed a clear trend towards increased thrombin activity (18.93 ± 10.61 mU/ml) compared to the control and RRD1 group, but this was not statistically significant. Thrombin activity (39.30 ± 14.04 mU/ml) was significantly ($P < 0.05$) elevated in the vitreous of patients with established PVR compared to all other groups (Figure 1). There were no statistical differences between the gender or age of the patients in the different groups (Table 1).

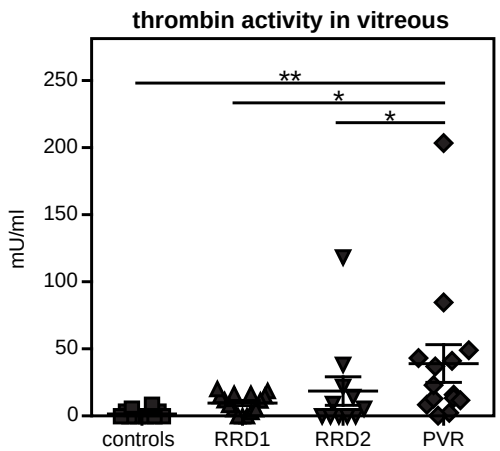

Figure 1: Thrombin activity in vitreous samples (controls: macular holes n= 3 and macular puckers n = 8, rhegmatogenous retinal detachment with no PVR development after vitrectomy (RRD1) n = 15, rhegmatogenous retinal detachment with PVR development after vitrectomy (RRD2) n = 11 and established PVR (PVR) n = 14) was determined with the thrombin-specific chromogenic substrate Tos-Gly-Pro-Arg-pNA in the absence and presence of the thrombin-specific inhibitor hirudin. Statistical analysis was performed by using a Kruskal-Wallis (One-way ANOVA) test followed by the Mann-Whitney U test. $P < 0.05$ was considered significant. *$P < 0.05$ and **$P < 0.01$.

The effect of different vitreous dilutions on IL6 and PDGFB mRNA expression by ARPE-19

Vitreous enhanced *IL6* mRNA expression by ARPE-19 cells after 1 and 2 hours of stimulation, which returned to baseline level again after 4 hours of stimulation (Figure 2). *PDGFB* mRNA expression level by ARPE-19 was also enhanced by vitreous stimulation with equal levels of induction after 1, 2 and 4 hours of stimulation (Figure 2). No differences between the 1/4 and 1/8 vitreous dilution were seen for either *IL6* mRNA or *PDGFB* mRNA induction in ARPE-19 (Figure 2). Based on these data further studies were undertaken with vitreous dilutions of 1/8.

The effects of vitreous on cytokine, chemokine and growth factor production by ARPE-19

Stimulation of ARPE-19 cells with RRD1, RRD2 and PVR vitreous samples for 24 hours significantly ($P < 0.05$) stimulated CCL2, CXCL8, IL-6, IL-12 (p70) and VEGF-A production by ARPE-19 cells (Figure 3). PVR vitreous stimulated CCL2, CXCL8 and IL-6 production by ARPE-19 to significantly ($P < 0.05$) higher levels than RRD1 and RRD2 vitreous samples,

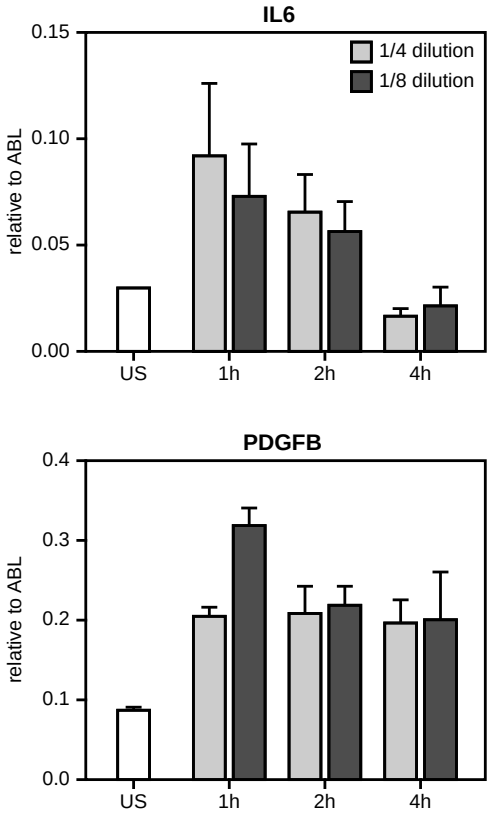

Figure 2: ARPE-19 cells were stimulated for 1, 2 and 4 hours with vitreous of patients with established PVR (4x and 8x diluted) and *IL6* (top panel) and *PDGFB* (bottom panel) mRNA expression levels were determined by RQ-PCR and normalized against the control gene *ABL*. Data are presented as the mean value from 4 independent experiments ± SEM. US = unstimulated.

while no differences were observed between the groups for vitreous-induced IL-12(p70) and VEGF-A production by ARPE-19. PVR vitreous stimulated G-CSF and IL-17A production by ARPE-19, albeit not to a statistically significant level. PDGF-BB production by ARPE-19 was significantly ($P < 0.05$) enhanced by PVR vitreous samples, but not by RRD1 and RRD2 vitreous samples (Figure 3). CCL4 levels were significantly higher ($P < 0.05$) in the T0 samples from RRD2 and PVR patients compared to T0 samples from RRD1 patients while CXCL10 was significantly ($P < 0.05$) higher in the T0 samples from PVR patients compared to T0 samples from RRD1 and RRD2 patients (Figure 3). CCL4 and CXCL10 levels did not increase after stimulating the ARPE-19 cells for 24 hours with vitreous from either the RRD1, RRD2 or PVR groups (Figure 3). In all groups basic fibroblast growth factor (FGFb) levels significantly ($P < 0.05$) declined after 24 hours of ARPE-19 stimulation when compared to the levels detected in the T0 samples (Figure 3). After 24 hours of ARPE-19 stimulation GM-CSF levels declined significantly ($P < 0.05$) in the RRD1 and the RRD2 groups but not in the PVR group, with GM-CSF levels being significantly ($P < 0.05$) higher in the PVR group compared to RRD1 and RRD2 samples at T24. All other factors measured by the used multiplex assay were not detected. For a total summary see supplemental table 1.

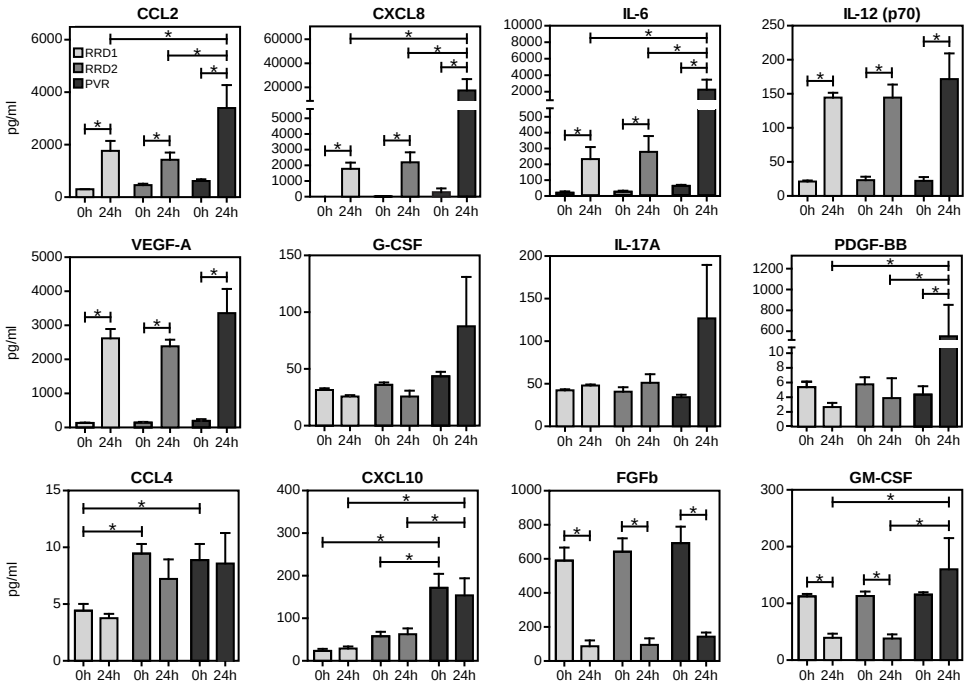

Figure 3: ARPE-19 cells were stimulated for 24 hours with 8x diluted vitreous from the RRD1, RRD2, PVR patient groups. Culture supernatants were analyzed by a Bio-Plex Pro™ Human Cytokine, Chemokine, and Growth Factor Assay; allowing the detection of 27 cytokines, chemokines and growth factors simultaneously. Data are presented as the mean value from 4 individual vitreous samples per group ± SEM. Statistical analysis was performed by using a Kruskal-Wallis (One-way ANOVA) test followed by the Mann-Whitney U test. $P < 0.05$ was considered significant. *$P < 0.05$.

The effect of thrombin inhibition on PVR vitreous-induced cytokine, chemokine and growth factor production by ARPE-19

Stimulation of ARPE-19 cells with PVR vitreous samples for 24 hours significantly ($P < 0.05$) stimulated CCL2, CXCL8, GM-CSF, IL-6 and PDGF-BB production by ARPE-19 cells compared to the RRD1 and RRD2 vitreous samples (Figure 3 and supplemental table 1). Thrombin (5 U/ml), used as positive control, also significantly ($P < 0.05$) stimulated CCL2, CXCL8, GM-CSF, IL-6 and PDGF-BB production by ARPE-19, which was completely abolished by the direct thrombin inhibitor hirudin ($P < 0.05$) (Figure 4 and supplemental table 1). The stimulatory effect of RRD1 and RRD2 vitreous on the production of these factors by ARPE-19 was not affected by hirudin (Figure 4 and supplemental table 1). Hirudin did significantly ($P < 0.05$) reduce the capacity of PVR vitreous to stimulate CCL2, CXCL8, GM-CSF, IL-6 and PDGF-BB production by ARPE-19 after 24 hours of stimulation (Figures 4 and supplemental table 1). This inhibition was not complete and reached the levels induced by the RRD1 and RRD2 vitreous samples (Supplemental table 1). Thrombin (5 U/ml) significantly ($P < 0.05$) stimulated *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* mRNA expression by ARPE-19 after 2 hours of stimulation, which was significantly ($P < 0.05$) reduced by hirudin (Figure 5). PVR vitreous samples also enhanced *CCL2*, *CXCL8*, *GMCSF, IL6* and *PDGFB* mRNA expression levels by ARPE-19 after 2 hours of stimulation, which was signi-

ficant ($P < 0.05$) for *CXCL8*, *IL6* and *PDGFB* mRNA (Figure 5). Addition of hirudin reduced the capacity of the PVR vitreous samples to stimulate *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* mRNA production by ARPE-19, which was significant ($P < 0.05$) for *CXCL8*, *IL6* and *PDGFB*.

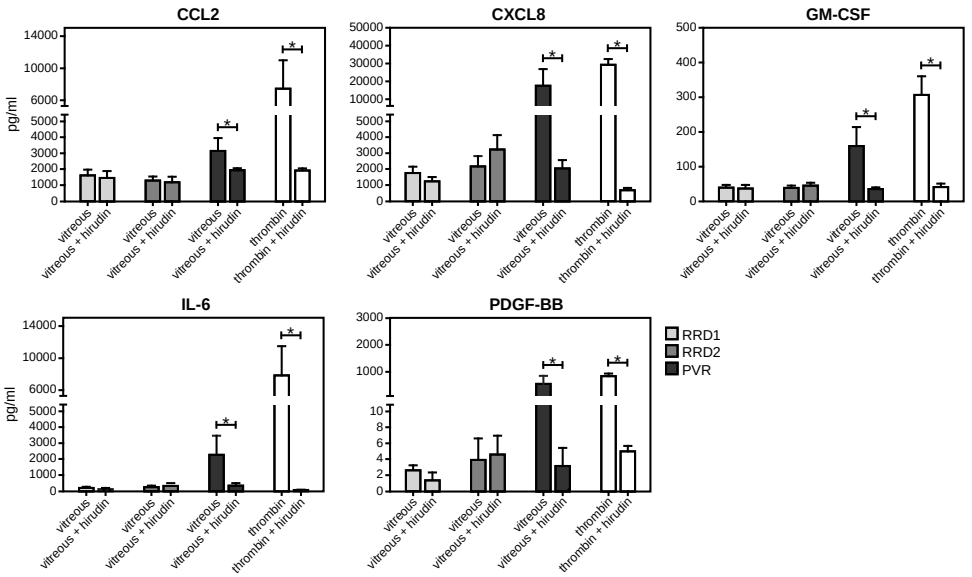

Figure 4: ARPE-19 cells were stimulated for 24 hours with 8x diluted vitreous from the RRD1, RRD2, PVR patient groups or thrombin (5 U/ml) in the absence or presence of the thrombin-specific inhibitor hirudin (7,5 U/ml). Culture supernatants were analyzed by a Bio-Plex Pro™ Human Cytokine, Chemokine, and Growth Factor Assay; allowing the detection of 27 cytokines, chemokines and growth factors simultaneously. Factors that showed significant differences between PVR, RRD1 and RRD2 after 24 hours of stimulation (Figure 3) are shown. Data are presented as the mean value from 4 individual samples per group ± SEM. Statistical analysis was performed by using a Kruskal-Wallis (One-way ANOVA) test followed by the Mann-Whitney U test. $P < 0.05$ was considered significant. *$P < 0.05$.

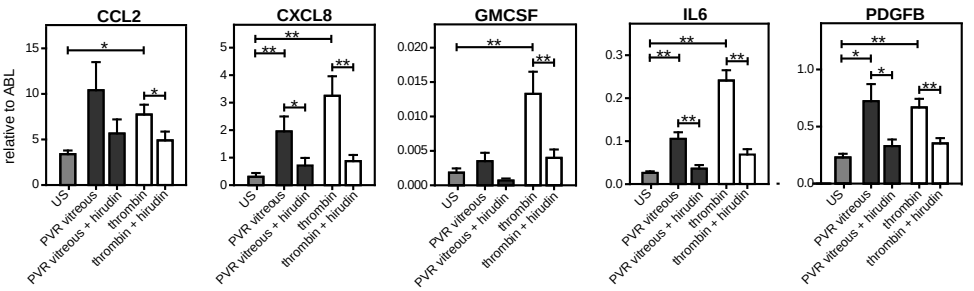

Figure 5: ARPE-19 cells were stimulated for 2 hours with 8x diluted vitreous from PVR patients (n = 7) or thrombin (5 U/ml) in the absence or presence of the thrombin-specific inhibitor hirudin (7,5 U/ml). *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* mRNA expression levels were determined by RQ-PCR and normalized against the control gene *ABL*. Data are presented as the mean value ± SEM. Statistical analysis was performed with the Kruskal-Wallis (one-way ANOVA) test followed by the Mann-Whitney U test when applicable. $P < 0.05$ was considered significant. *$P < 0.05$ and **$P < 0.01$. US = unstimulated.

Discussion

Breakdown of the blood-retinal barrier is associated with PVR and a role for direct RPE activating effects of thrombin have been proposed to contribute to the pathobiology of PVR. Our study is the first to demonstrate increased thrombin activity in vitreous from patients with established PVR and that this stimulates RPE cells to produce pro-inflammatory cytokines and chemokines such as CCL2, CXCL8, GM-CSF and IL-6, as well as the pro-fibrotic mediator PDGF-BB.

In this study we measured thrombin activity in vitreous from patients with macular hole development, idiopathic macular pucker formation, rhegmatogenous retinal detachment without PVR development after vitrectomy, rhegmatogenous retinal detachment with later PVR development after vitrectomy and established PVR. In macular hole and macular pucker patients the blood retinal barrier is intact and we detected no-to-low thrombin activity in these vitreous samples, with levels comparable to that reported in two recently published studies [20, 21]. Compared to this, thrombin activity was significantly elevated in vitreous from patients with established PVR, while a trend towards increased thrombin activity was seen in vitreous from patients that developed PVR within six months following vitrectomy. Our study therefore supports that PVR development is a gradual process in which later stages of disease are associated with increased intravitreal thrombin activity. Whether this increase is cause or consequence of disease progression is so far unclear. Nevertheless, measurement of intraocular thrombin activity might represent a new biomarker for PVR development that warrants further studies.

Inflammation is an important component of PVR pathogenesis and RPE cells contribute to ocular inflammation via the production of pro-inflammatory mediators. Thrombin has been described as a potent inducer of cytokine/chemokine and growth factor production by RPE cells via activation of PAR1 [10, 12]. Here we found that vitreous from the RRD1, RRD2 and PVR groups significantly stimulated the production of CCL2, CXCL8 (IL-8), IL-6, IL-12 (p70) and VEGF-A by RPE-cells. Vitreous of patients with established PVR stimulated the production of CCL2, CXCL8, and IL-6 by RPE cells to significantly higher levels than vitreous from the RRD1 and RRD2 groups. Thrombin inhibition only inhibited the capacity of PVR vitreous to induce CCL2, CXCL8, and IL-6 production by ARPE-19, but although significant this was not complete and reached the levels induced by the RRD1 and RRD2 vitreous samples (Supplemental table 1). Thus in established PVR, intravitreal thrombin activity is a major, but not the sole, factor that stimulates production of the pro-inflammatory mediators CCL2, CXCL8 and IL-6 by RPE, while in RRD patients without PVR (RRD1) development or PVR development later on (RRD2) the induction of these factors appears to be largely independent of intravitreal thrombin activity. The vitreous-induced production of VEGF-A and IL-12 (p70) was equal between the three groups and not reduced by thrombin inhibition (Supplemental table 1), suggesting no or a limited role for intravitreal thrombin in inducing the production of these factors by ARPE-19. Thrombin (5 U/ml) did however induce IL-12 (p70) and VEGF-A by ARPE-19 (Supplemental table 1), while others demonstrated a dose-dependent effect of thrombin on VEGF-A production [12]. In our experiments we used diluted vitreous (1/8), which may have obscured effects of intravitreal thrombin activity on the production of factors such as IL-12 (p70), VEGF-A as well as others by the ARPE-19 cells. Incubation of the ARPE-19 cells with vitreous from RRD1 and RRD2 patients resulted in a decline of GM-CSF levels in the culture media. This might be related to binding of GM-CSF to its receptor, which

is ubiquitous expressed by epithelial cells [22]. This decline in GM-CSF was not observed when PVR vitreous was added to the ARPE-19 cells. Moreover thrombin did stimulate GM-CSF secretion by ARPE-19 (Supplemental table 1), while PVR vitreous enhanced GM-CSF mRNA levels in RPE cells, as did thrombin alone, which was blocked by hirudin. This indicates that intravitreal thrombin activity present in PVR vitreous stimulates GM-CSF production by RPE cells. Our data therefore clearly implicate that intravitreal thrombin activity is involved in driving the production of CCL2, CXCL8, GM-CSF and IL-6 by RPE in PVR. CCL2, CXCL8, GM-CSF and IL-6 are potent activators and chemoattractants for immune cells such as monocytes, macrophages, neutrophils and B-lymphocytes which are present in PVR membranes and vitreous, while GM-CSF also stimulates differentiation of monocytes into macrophages [10, 23-26].

Retinal fibrotic contractile membranes represent the final stage of PVR, but their formation requires a complex process of proliferation, migration, de-differentiation and extracellular matrix deposition by different cell types, amongst which RPE, that is driven by pro-fibrotic mediators [1]. Basic fibroblast growth factor (FGFb) is a pro-fibrotic factor proposed to contribute to PVR [27]. We found a reduction of FGFb after vitreous incubation with ARPE-19 while thrombin alone did not induce FGFb production by ARPE-19 (Supplemental table 1) [10]. This suggests that the reduction in FGFb might be due to binding to its receptor which is expressed by RPE and illustrates that thrombin does not stimulate FGFb production by RPE cells. PDGF is considered an important pro-fibrotic mediator in PVR that induces proliferation and de-differentiation of RPE cells into contractile myofibroblasts that are abundantly present in PVR membranes and represent the main cellular subset responsible for excessive collagen production in fibrosis [4, 27-30]. Recently, we demonstrated that thrombin induces de-differentiation of RPE cells into collagen producing myofibroblasts via autocrine release of PDGF-BB [9]. Here we found that vitreous from PVR patients strongly stimulated the production of PDGF-BB by ARPE-19 in a thrombin dependent manner. Consequently elevated intravitreal thrombin activity can activate pro-fibrotic processes in PVR, at least via autocrine PDGF-BB release and signaling in RPE cells.

Vitreous samples did not stimulate production of the chemokines CCL4 and CXCL10 by ARPE-19 cells. CCL4 and CXCL10 were, however, present at higher levels in the prepared 1/8 diluted vitreous samples (T0, Figure 3 and supplemental table 1) from the patients that later on developed PVR (RRD2) or had established PVR at the time of vitrectomy. Vitreous from established PVR contained higher levels of CXCL10 than RRD2 vitreous, implicating that CXCL10 has a more profound role in later stages of disease. These observations are in line with previous reports and support a role for these chemokines in the inflammatory response in PVR and suggest that CCL4 and CXCL10 may represent ocular biomarkers for PVR development [31-33].

Polarity is an important determinant for RPE function in the maintenance of ocular homeostasis [34]. For our studies we used non-polarized ARPE-19 monolayers. It has however been described that polarized RPE can react differently under inflammatory conditions than non-polarized cells with regard to pigment epithelial derived factor and VEGF production, which is related to opposing activity levels of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and c-Jun N-terminal kinase (JNK) pathway [34, 35]. Previously we demonstrated that thrombin-induced cytokine/chemokine/

growth factor production by ARPE-19 and primary RPE was mediated via PAR1-induced NF-κB signaling, while others demonstrated that thrombin activates multiple signaling pathways post-PAR1, including NF-κB and JNK [10, 11]. Therefore we cannot exclude the possibility that polarized ARPE-19 cells would have responded differently with regard to vitreous-induced cytokine/chemokine/growth factor production compared to non-polarized ARPE-19 cells, for instance for VEGF production. However, RPE dedifferentiation and loss of polarity is part of PVR development and progression [36].
In conclusion, this study demonstrates that PVR development is associated with an increase in intravitreal thrombin activity, especially during the later stage of PVR, and supports a role for intravitreal thrombin activity in stimulating inflammatory and fibrotic pathways in RPE. We propose a comparable role for thrombin in other inflammatory proliferative vitreoretinal disorders with blood retinal barrier breakdown, such as exudative age-related macular degeneration, proliferative diabetic retinopathy and retinal vein occlusion, of which the latter was recently found to be associated with increased intravitreal thrombin activity [22]. Inhibition of thrombin activity may therefore represent a potential treatment option for proliferative vitreoretinal diseases as PVR.

Acknowledgments

The authors thank Sandra de Bruin – Versteeg for her help with the illustrations.

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Supplemental table 1 : Bio-Plex Pro™ Human Cytokine, Chemokine, and Growth Factor Assay analysis

	PBS		PBS + hirudin		RRD1		RRD1 + hirudin		RRD2	
	To	T24	To	T24	To	T24	To	T24	To	T24
IL-1β	not detectable									
IL-1rα	not detectable									
IL-2	not detectable									
IL-4	not detectable									
IL-5	not detectable									
IL-6	1.21 ± 0.14	40.57 ± 12.22	1.99 ± 0.36	67.07 ± 3.62	15.18 ± 8.67	229.67 ± 75.92	20.28 ± 13.38	158.43 ± 63.43	21.80 ± 6.13	275.73 ± 102.27
IL-7	not detectable									
IL-8/CXCL8	9.30 ± 1.34	277.82 ± 88.37	9.33 ± 1.34	475.38 ± 59.96	8.53 ± 0.32	1783.76 ± 390.58	9.97 ± 1.20	1269.07 ± 263.88	12.52 ± 1.69	.63 ± 650.69
IL-9	11.97 ± 1.58	5.01 ± 0.63	13.23 ± 2.11	4.57 ± 0.59	10.02 ± 0.51	6.09 ± 1.30	11.98 ± 1.80	5.67 ± 0.90	11.55 ± 1.16	7.82 ± 2.07
IL-10	not detectable									
IL-12 (p70)	16.15 ± 4.07	112.52 ± 12.46	27.61 ± 5.29	123.47 ± 5.05	21.30 ± 1.57	144.77 ± 6.50	24.10 ± 3.25	153.90 ± 11.00	23.28 ± 4.68	144.17 ± 19.72
IL-13	0.61 ± 0.09	1.18 ± 0.19	0.82 ± 0.23	1.31 ± 0.08	1.68 ± 0.40	2.21 ± 0.24	1.84 ± 0.48	1.86 ± 0.15	2.88 ± 0.75	2.58 ± 0.70
IL-15	not detectable									
IL-17	52.73 ± 6.59	42.71 ± 11.83	63.69 ± 9.07	49.65 ± 3.92	42.42 ± 1.23	48.02 ± 1.29	50.65 ± 4.58	49.56 ± 4.22	40.70 ± 5.08	51.24 ± 10.25
FGFb	473.37 ± 162.29	93.92 ± 33.97	623.21 ± 57.99	115.88 ± 9.16	590.20 ± 77.10	86.81 ± 34.26	607.90 ± 61.22	43.17 ± 7.18	642.52 ± 77.50	93.40 ± 38.61
Eotaxin	not detectable									
G-CSF	29.88 ± 9.50	21.72 ± 6.33	37.60 ± 4.95	25.67 ± 1.66	31.52 ± 1.30	25.69 ± 1.15	42.05 ± 3.81	25.14 ± 0.99	35.79 ± 2.17	25.70 ± 5.07
GM-CSF	148.44 ± 24.54	43.96 ± 11.88	140.49 ± 10.44	59.19 ± 10.70	112.43 ± 3.84	39.27 ± 7.40	125.44 ± 8.12	37.63 ± 9.00	113.22 ± 7.91	38.19 ± 7.24
IFNγ	6.49 ± 0.65	16.79 ± 3.72	9.88 ± 1.92	20.46 ± 1.00	6.87 ± 0.41	23.17 ± 1.26	8.67 ± 1.48	23.03 ± 1.97	6.80 ± 1.51	25.06 ± 3.73
IP-10/CXCL10	2.52 ± 0.28	4.60 ± 1.32	4.08 ± 1.06	5.08 ± 0.42	23.58 ± 5.04	28.81 ± 4.89	26.97 ± 5.53	27.64 ± 5.31	58.10 ± 9.97	62.91 ± 13.57
MCP-1/CCL2	107.23 ± 30.23	1618.93 ± 186.16	192.47 ± 26.89	2148.21 ± 240.98	275.88 ±8.18	1633.06 ± 352.4 5	287.53 ± 16.29	1474.68 ± 419.83	427.66 ± 40.76	1316.33 ± 256.85
MIP-1α/CCL3	not detectable									
MIP-1β/CCL4	1.92 ± 1.21	1.42 ± 0.42	2.60 ± 0.77	2.03 ± 0.13	4.45 ± 0.56	3.78 ± 0.37	5.50 ± 0.76	3.57 ± 0.52	9.47 ± 0.82	7.21 ± 1.74
PDGF-BB	3.32 ±0.22	3.15 ±1.64	3.40 ±1.48	2.95 ±1.00	5.40 ± 0.75	2.71 ± 0.39	5.58 ± 1.21	1.40 ± 0.70	5.79 ± 1.00	3.92 ± 1.91
RANTES	not detectable									
TNF-α	not detectable									
VEGF-A	122.19 ± 21.34	1840.05 ± 276.47	186.26 ± 25.49	2274.15 ± 51.80	140.69 ± 4.77	2627.33 ± 271.35	151.74 ± 12.02	2554.81 ± 311.97	148.32 ± 23.33	2386.72 ± 188.63

All cytokines/chemokines and growth factors detectable with the Bio-Plex Pro™ Human Cytokine, Chemokine and Growth Factor
lated situation (PBS) or after stimulation with vitreous or thrombin (5 U/ml) both in the presence and absence of hirudin is indicated

	RRD2 + hirudin		PVR		PVR + hirudin		thrombin		thrombin + hirudin		LLOQ	ULOQ
	To	T24	To	T24	To	T24	To	T24	To	T24		
											3.2	3261
											81.1	70487
											2.1	17772
											2.2	3467
											3.1	7380
	24.34 ± 7.30	357.87 ± 151.82	57.75 ± 7.9	2267.63 ± 1208.89	59.22 ± 9.19	368.69 ± 138.98	36.47 ± 14.98	7830.13 ± 3580.09	2.77 ± 1.00	105.84 ± 18.72	2.3	18880
											3.1	6001
	14.18 ± 2.25	3235.38 ± 903.7	271.32 ±250.33	17493.14± 9475.47	19.02 ± 2.57	2058.82 ± 532.18	802.13 ± 292.78	29362.23 ± 3207.66	8.95 ± 2.1	700.67 ± 137.69	1.9	26403
	13.96 ± 1.11	9.12 ± 2.01	10.43 ± 0.64	12.16 ± 3.81	15.00 ± 2.84	2.63 ± 0.85	13.76 ± 1.26	16.69 ± 1.22	15.47 ± 4.26	5.18 ± 1.23	2.1	7989
											2.2	8840
	25.24 ± 4.69	164.03 ± 16.24	22.02 ± 5.85	171.62 ± 37.97	26.43 ± 3.07	122.75 ± 3.59	38.33 ± 4.99	205.73 ± 4.27	32.73 ± 5.32	113.22 ± 7.10	3.3	13099
	3.07 ± 0.71	2.99 ± 0.73	4.67 ± 1.37	3.98 ± 1.10	3.72 ± 0.89	2.5 ± 0.47	0.55 ± 0.19	2.7 ± 0.10	0.92 ± 0.34	1.31 ± 0.19	3.7	3137
											2.1	2799
	46.56 ± 4.90	60.64 ± 9.67	34.43 ± 2.74	126.88 ± 62.93	47.92 ± 8.92	46.67 ± 3.02	52.23 ± 11.13	117.12 ± 9.30	67.27 ± 16.48	48.91 ± 7.97	4.9	12235
	715.43 ± 51.94	119.78 ± 46.26	692.40 ± 96.83	143.19 ± 23.37	697.10 ± 76.55	55.77 ± 11.55	692.23 ± 183.86	405.80 ± 139.88	712.71 ± 157.80	91.51 ± 29.71	40.9	5824
											27.2	7581
	39.43 ± 3.26	29.15 ± 4.10	43.49 ± 4.01	87.50 ± 43.57	58.67 ± 10.93	31.96 ± 2.84	23.99 ± 5.15	77.48 ± 6.65	37.59 ± 8.32	25.73 ± 2.92	2.4	11565
	124.98 ± 6.84	45.88 ± 8.17	115.49 ± 3.86	135.15 ± 66.11	131.00 ± 13.90	36.21 ± 4.52	146.68 ± 12.62	307.12 ± 53.74	158.14 ± 22.51	41.75 ± 10.27	63.3	6039
	8.15 ± 0.71	27.17 ± 3.93	7.60 ± 1.34	44.36 ± 17.51	9.24 ± 1.90	20.81 ± 1.43	9.30 ± 2.91	50.48 ± 3.73	10.75 ± 2.65	20.24 ± 3.02	92.6	52719
	78.88 ± 16.26	66.74 ± 13.76	172.51 ± 33.14	154.78 ± 40.32	212.64 ± 56.58	150.89 ± 24.43	4.83 ± 0.56	13.07 ± 0.78	4.13 ± 1.70	4.93 ± 1.60	18.8	26867
	429.90 ± 39.60	1216.11 ± 324.46	560.76 ± 67.02	3365.65 ± 729.62	445.41 ± 38.13	1957.86 ±117.37	443.19 ± 112.32	7468.30 ± 3529.43	212.52 ± 22.93	1936.54 ± 123.48	2.1	1820
											1.4	836
	10.86 ± 0.77	7.92 ± 1.76	8.87 ± 1.44	8.59 ± 2.69	10.05 ± 2.73	4.88 ± 0.65	3.05 ± 0.79	6.52 ± 0.45	3.51 ± 1.09	2.94 ± 0.32	2.0	1726
	6.51 ± 1.12	4.65 ± 1.97	4.39 ± 1.16	553.92 ± 260.17	6.78 ± 1.54	3.22 ± 1.59	7.43 ±1.47	853.28 ±80.73	6.38 ±2.59	5.03 ±0.46	7.0	51933
											2.2	8617
											5.8	95484
	156.29 ± 19.41	2442.43 ± 159.90	200.88 ± 50.69	3366.20 ± 712.86	177.16 ± 12.21	2611.92 ± 66.76	350.07 ± 50.71	4989.78 ± 254.25	230.76 ± 27.41	2333.01 ± 125.82	5.5	56237

Assay are given in the first column. The detected concentration of these factors in culture supernatants from ARPE-19 cells in unstimu-
in the second till twentieth column, at t = 0 hours (To) and t = 24 hours (T24).

Chapter 6

Dabigatran inhibits thrombin and PVR vitreous-induced proinflammatory and profibrotic mediator production by ARPE-19 cells

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Submitted for publication

Abstract

Purpose: Proliferative vitreoretinopathy (PVR) is a vitreoretinal inflammatory fibrotic disorder characterized by the formation of fibroproliferative membranes. Retinal pigment epithelial (RPE) cells contribute to vitreoretinal inflammation and fibrosis in PVR. Vitreous of PVR patients contains increased thrombin activity which induces proinflammatory and profibrotic programs in RPE cells. Inhibition of intravitreal thrombin activity should therefore be considered as treatment option for PVR. In this study we examined the capacity of the clinically available direct thrombin-inhibitor dabigatran and the clinically available anti-inflammatory agent dexamethasone to inhibit thrombin- and PVR vitreous-induced expression of inflammatory/fibrotic mediators by ARPE-19 cells.

Methods: ARPE-19 and HFL-1 cells were cultured with thrombin in the presence or absence of dabigatran (range: 10^{-5} - 10^{-7} M), dexamethasone (range: 10^{-7} - 10^{-9} M) or the NF-κB inhibitor SC-514 (10^{-4} M). Moreover, ARPE-19 cells were cultured with vitreous lacking thrombin activity and vitreous from patients with PVR with elevated thrombin activity in the presence and absence of dabigatran (10^{-5} M). *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* mRNA expression levels were determined by RQ-PCR.

Results: Thrombin and PVR vitreous induced *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* expression by ARPE-19 cells, which was inhibited by dabigatran. Vitreous without thrombin activity had no effect on *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* expression levels by ARPE-19 cells. Dexamethasone did not inhibit the effect of thrombin on ARPE-19 cells.

Conclusions: Our findings indicate that dabigatran is capable to inhibit RPE activation due to increased intravitreal thrombin activity associated with PVR.

Introduction

Thrombin is a key serine protease of blood coagulation that converts soluble fibrinogen into insoluble fibrin [1]. In addition to its role in coagulation, thrombin induces cellular responses that have important roles in inflammation and tissue repair, but also contribute to the development of fibrosis [2-4]. Thrombin for instance stimulates the production of inflammatory mediators by several cell types, promotes chemotaxis of inflammatory cells, stimulates the proliferation of fibroblasts and smooth muscle cells, and induces myofibroblast differentiation and the production extracellular matrix (ECM) components such as collagen [5]. These cellular responses to thrombin are mostly mediated via the G protein-coupled receptor protease-activated receptor (PAR)-1 [6].

Proliferative vitreoretinopathy (PVR) is a complication of retinal detachment caused by a retinal tear (rhegmatogenous retinal detachment (RRD)) or trauma that is characterized by inflammation and the formation of subretinal, intraretinal, and/or epiretinal fibrotic membranes [7, 8]. Retinal pigment epithelial (RPE) cells dispersed during retinal detachment, are important contributors to PVR pathogenesis as they produce cytokines and chemokines that recruit and activate immune cells [7]. Moreover, dedifferentiation of these dispersed RPE cells into collagen producing myofibroblasts is a key event in the development of the contractile fibrotic membranes [8]. Although these pathobiological processes are well recognized to contribute to PVR, medical treatment options are so far limited and treatment mostly still depends on (recurrent) surgical intervention [8].

Activation of the coagulation cascade, as evidenced by intraocular fibrin deposition and increased intravitreal thrombin activity, occurs in PVR [9-11]. Moreover, we demonstrated that this increased intravitreal thrombin activity stimulates RPE cells to produce pro-inflammatory mediators (CCL2, CXCL8, GM-CSF, IL-6) that are involved in immune cell activation, recruitment and differentiation as well as the pro-fibrotic mediator platelet-derived growth factor (PDGF)-BB that subsequently induces dedifferentiation of RPE cells into collagen producing myofibroblasts [9, 12]. All together this data points at an important role for thrombin in stimulating pro-inflammatory and pro-fibrotic responses by RPE in PVR. Thrombin may therefore represent an attractive treatment target for this disease.

Dabigatran-etexilate is a small molecule oral pro-drug that is hydrolyzed to its active compound dabigatran. Dabigatran is a selective, competitive and reversible direct thrombin inhibitor (DTI) that binds to the active site of the thrombin molecule, thereby blocking the interaction between thrombin and its substrates [13]. Dabigatran prevents thrombin-induced cleavage of fibrinogen's fibrinopeptides at particular Arg-Gly bonds thereby precluding fibrin formation, but also inhibits thrombin induced cleavage of PAR-1 at the peptide bond between residues Arg-41 and Ser-42 [14, 15].

The present study was undertaken to investigate whether dabigatran inhibits thrombin-induced cytokine and growth factor production by RPE cells and to compare this with the effect established by the glucocorticoid dexamethasone, which was already used in clinical studies to modulate PVR. In addition, the efficacy of dabigatran to inhibit thrombin activity in vitreous of patients with established PVR was examined. We show that dabigatran inhibits thrombin-induced CCL2, CXCL8, GM-CSF, IL-6 and PDGF-

BB production by ARPE-19 cells, while dexamethasone did not. Moreover, dabigatran inhibited the capacity of PVR vitreous to induce CCL2, CXCL8, GM-CSF, IL-6 and PDGF-BB production by ARPE-19 cells. Therefore dabigatran may be considered as drug to treat PVR with the potential to reduce thrombin-induced proinflammatory and profibrotic mediator production by RPE.

Materials and Methods

Vitreous fluids

Vitreous samples without thrombin activity (rhegmatogenous retinal detachment (RDD) without PVR development after vitrectomy (n = 5)) and with thrombin activity (established PVR (n = 5): mean intravitreal thrombin activity = 56,19 ± 83,62 mU/ml) were selected from our previous study [9]. All subjects gave their consent for the use of rest material for research; storage and use of the vitreous for further studies were according to the guidelines of the Medical Ethics Committee of the Erasmus MC, University Medical Center, Rotterdam and were performed in accordance with guidelines established by the Declaration of Helsinki.

Cell cultures

The human retinal pigment epithelial cell line ARPE-19 and the human fetal lung fibroblast cell line HFL-1 were obtained from American Type Culture Collection (ATCC; Manassas, VA, US). ARPE-19 cells were cultured in RPE medium (Dulbecco's modified Eagle's medium (DMEM)/F-12 (HyClone, Logan, UT, US), containing 10% heat inactivated fetal calf serum (FCS) and penicillin/streptomycin (all from BioWhittaker, Verviers, Belgium)) and between passage 23-30 when used for experiments. HFL-1 cells were cultured in DMEM (BioWhittaker) containing 10% heat inactivated FCS and penicillin/streptomycin and between passage 14-18 when used for experiments. The cell lines were analyzed by short tandem repeat (STR) analysis (DSMZ, Braunschweig, Germany) for cell line authentication and were mycoplasma free. The cells lines were maintained under standard cell culture conditions at 37 °C in humidified air with 5% CO_2.

Determining the efficacy of dabigatran and dexamethasone to inhibit thrombin-induced cytokine and growth factor mRNA expression by ARPE-19 and HFL-1

ARPE-19 cells were seeded in 12-wells plates at a density of 3 x 10^5 cells/well in RPE medium containing 10% FCS and allowed to adhere overnight. Subsequently the medium was refreshed twice a week till the cells were grown 100% confluent. Prior to stimulation, the cells were serum starved in RPE medium containing 1% FCS for 24 hours followed by an additional 24 hours in serum free RPE medium. Hereafter cells were pre-incubated with fresh serum free RPE medium with or without active dabigatran (BIBR 953 range: 10^{-5} - 10^{-7} M: kindly provided by Boehringer Ingelheim Pharma GmbH & Co. KG (Ingelheim am Rein, Germany) or dexamethasone (range: 10^{-7} - 10^{-9} M; Centrafarm B.V., Etten-Leur, The Netherlands) for 60 minutes. Subsequently thrombin (Calbiochem, La Jolla, CA) was added to a final concentration of 5 U/ml (concentration based on previous studies [9, 12, 16]) or cells remained unstimulated for a period of 2 hours. Hereafter cells were harvested for RNA isolation. Experiments were also performed with the human fetal lung fibroblast cell line HFL-1, which was seeded in 12-wells plates at a density of 2 x 10^5 cells/well in DMEM containing 10% FCS and allowed to adhere overnight. Prior to stimulation, the cells were serum starved in DMEM containing 1% FCS for 24 hours followed by an additional 24 hours in serum free DMEM. The dabigatran and dexamethasone concentrations used were nontoxic to ARPE-19 and HFL-1 cells as determined by lactate dehydrogenase (LDH) release (Roche, Mannheim, Germany) and microscopic appearance of the cells. In addition, inhibition experiments were performed with the NF-κB inhibitor SC-514 (10^{-4} M; Calbiochem, concentration based on previous studies [16, 17]).

Determining the efficacy of dabigatran to inhibit PVR vitreous-induced cytokine, chemokine and growth factor mRNA expression levels in ARPE-19

ARPE-19 cells were seeded in 12-wells plates at a density of 3 x 10^5 cells/well in RPE medium containing 10% FCS and allowed to adhere overnight. Subsequently the medium was refreshed twice a week till the cells were grown 100% confluent. Prior to stimulation, the cells were serum starved in RPE medium containing 1% FCS for 24 hours followed by an additional 24 hours in serum free RPE medium. Hereafter, cells were pre-incubated with serum free RPE medium with or without dabigatran (10^{-5} M) for 60 minutes and subsequently stimulated for 2 hours with serum free medium containing 1/8 diluted vitreous (dilution based on previous study [9]) with or without dabigatran (10^{-5} M). Hereafter cells were harvested for RNA isolation.

RNA isolation and real-time quantitative PCR

RNA was isolated using a GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO) and reverse transcribed into cDNA [9]. Transcript levels of *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* mRNA were determined by real-time quantitative (RQ)-PCR (7700 PCR system; Applied Biosystems [ABI], Foster City, CA, US) using primer and probe combinations as previously described [9]. In addition the expression level of the glucocorticoid receptor gene *NR3C1* was determined in unstimulated cells using a commercially available Taqman Gene Expression assay (Applied Biosystems). Expression levels of the analyzed gene transcripts were normalized to the control gene *ABL* (Abelson) [9].

Statistical analysis

Messenger RNA data were analyzed using the Kruskal-Wallis (One-way ANOVA) test followed by the Mann-Whitney U test when applicable. A *P*-value < 0.05 was considered significant.

Results

Effect of dabigatran and dexamethasone on thrombin-induced cytokine, chemokine and growth factor mRNA expression by ARPE-19

In line with our previous observations, thrombin significantly ($P < 0.05$) induced *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* mRNA expression levels in ARPE-19 (Figure 1) [9, 12, 16]. This stimulatory effect of thrombin was dose-dependently inhibited by dabigatran for all genes, being significant ($P < 0.05$) at a concentration of 10^{-5} M (Figure 1A). In contrast, dexamethasone at the tested concentrations did not inhibit the thrombin-induced increase in *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* mRNA levels in ARPE-19 (Figure 1B). Dabigatran and dexamethasone alone had no effect on *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* mRNA expression levels (Figure 1).

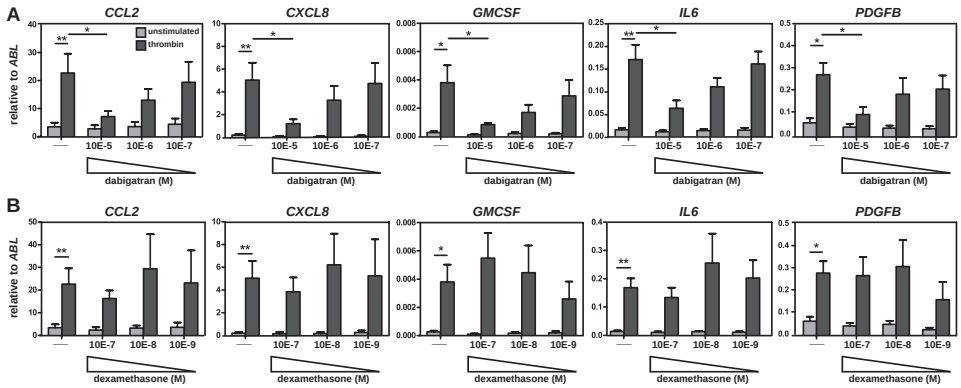

Figure 1: ARPE-19 cells were stimulated for 2 hours with thrombin (5 U/ml) or remained unstimulated in the absence or presence of (**A**) dabigatran (range: 10^{-5} - 10^{-7} M) or (**B**) dexamethasone (range: 10^{-7} - 10^{-9} M). *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* mRNA expression levels were determined by RQ-PCR and normalized against the control gene *ABL*. Data are presented as the mean value from 4 independent experiments ± SEM. Statistical analysis was performed with the Kruskal-Wallis (One-way ANOVA) test followed by the Mann-Whitney U test when applicable. $P < 0.05$ was considered significant. ***** = $P < 0.05$ and ****** = $P < 0.01$.

Effects of dabigatran and dexamethasone on thrombin-induced cytokine, chemokine and growth factor mRNA expression by ARPE-19 and HFL-1

In order to determine whether the inability of dexamethasone to inhibit the thrombin-induced increase in *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* mRNA levels was confined to ARPE-19 we also examined the response of the human fetal lung fibroblast cell line HFL-1. Because we previously found that thrombin-induced cytokine/chemokine and growth factor production by RPE cells involves activation of the NF-κB pathway, which is also a target for the glucocorticoid/glucocorticoid receptor complex, we used the specific NF-κB inhibitor SC-514 as control in these experiments [16, 18]. Thrombin stimulation enhanced *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* mRNA expression levels both in ARPE-19 and HFL-1 and this effect was significantly inhibited ($P < 0.05$) up to basal expression levels by dabigatran in both cell lines (Figure 2). Dexamethasone did not inhibit the effects of thrombin in ARPE-19 but did inhibit ($P < 0.05$) the thrombin-induced increase in *CCL2*,

CXCL8, *GMCSF* and *IL6* mRNA levels in HFL-1, not the increase in *PDGFB* mRNA (Figure 2). SC-514 inhibited the thrombin-induced increase in *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* mRNA levels in both cell lines.

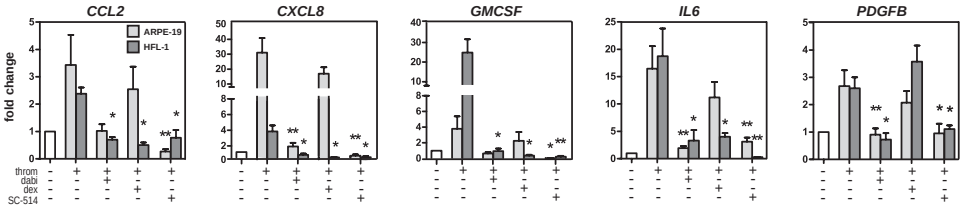

Figure 2: ARPE-19 and HFL-1 cells were stimulated for 2 hours with thrombin (5 U/ml) or remained unstimulated in the absence or presence of dabigatran (dabi, 10^{-5} M) dexamethasone (dex, 10^{-7} M) or SC-514 (10^{-4} M). *CCL2*, *CXCL8, GMCSF, IL6* and *PDGFB* mRNA expression levels were determined by RQ-PCR and normalized against the control gene *ABL*. Data are presented as the mean value from 4-5 independent experiments ± SEM. Statistical analysis was performed with the Kruskal-Wallis (One-way ANOVA) test followed by the Mann-Whitney U test when applicable. $P < 0.05$ was considered significant. * = $P < 0.05$ and ** = P 0.01.

Glucocorticoid receptor mRNA expression by ARPE-19 and HFL-1 cells

Because the above data indicate a relative unresponsiveness of ARPE-19 to dexamethasone we examined the mRNA expression level of the glucocorticoid receptor encoding gene *NR3C1*. This revealed that *NR3C1* mRNA was expressed at a significantly ($P < 0.05$) higher level in HFL-1 than in ARPE-19 (Figure 3). This suggests that the unresponsiveness of ARPE-19 to dexamethasone may be related to low glucocorticoid receptor expression.

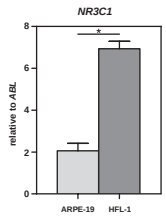

Figure 3: *NR3C1* mRNA expression levels were determined in unstimulated ARPE-19 and HFL-1cells by RQ-PCR and normalized against the control gene *ABL*. Data are presented as the mean value from 6 independent measurements ± SEM. Statistical analysis was performed with the Mann-Whitney U test. $P < 0.05$ was considered significant. * = $P < 0.05$.

The effect of dabigatran on vitreous-induced cytokine, chemokine and growth factor mRNA expression by ARPE-19

Vitreous samples from RDD patients that lacked detectable thrombin activity did not enhance *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* mRNA expression by ARPE-19, which is in line with our previous observations [9]. Nor was this affected by dabigatran (Figure 4A). Vitreous samples from PVR patients that contained thrombin activity clearly enhanced *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* mRNA expression by ARPE-19, in line with our previous observations [9]. Dabigatran did significantly ($P < 0.05$) reduce the capacity of the PVR vitreous to stimulate *CCL2*, *CXCL8*, *IL6* and *PDGFB* mRNA expression by ARPE-19 (Figure 4B).

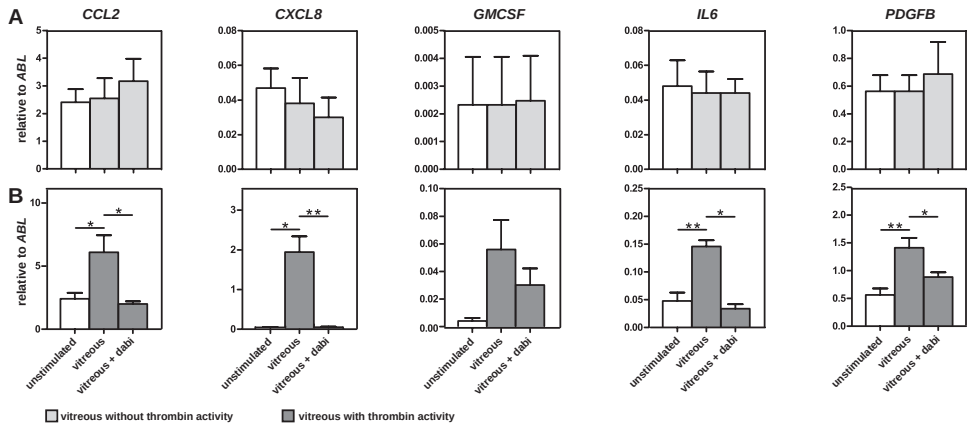

Figure 4: ARPE-19 cells were stimulated for 2 hours with (**A**) 1/8 diluted vitreous without thrombin activity or (**B**) 1/8 diluted vitreous with thrombin activity in the absence or presence of dabigatran (dabi, 10^{-5} M). *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* mRNA expression levels were determined by RQ-PCR and normalized against the control gene *ABL*. Data are presented as the mean value from 4 independent experiments ± SEM. Statistical analysis was performed with the Kruskal-Wallis (One-way ANOVA) test followed by the Mann-Whitney U test when applicable. $P < 0.05$ was considered significant. ***** = $P < 0.05$ and ****** = $P < 0.01$.

Discussion

There is accumulating evidence that elevated intravitreal thrombin activity is involved in the pathogenesis of PVR and that its contribution goes beyond fibrin formation. In PVR thrombin activates PAR-1 signaling in RPE cells resulting in production of the proinflammatory cytokines CCL2, CXCL8, GM-CSF, IL-6, the profibrotic mediator PDGF-BB and dedifferentiation of RPE cells into collagen producing myofibroblasts [12, 16]. Inhibition of thrombin may thus represent an attractive therapeutic option for PVR treatment. Our current findings demonstrate that dabigatran inhibits thrombin and PVR vitreous-induced elevation of *CCL2*, *CXCL8*, *GMCSF*, *IL6* and *PDGFB* mRNA levels in ARPE-19 cells. Previously we found a positive correlation for these mRNAs with the actual protein production when ARPE-19 were stimulated with thrombin or PVR vitreous indicating that the mRNA results can be translated to the protein level [9, 16].

Thrombin stimulates the production of CCL2, CXCL8, GM-CSF, IL-6 and PDGF-BB by RPE via PAR-1 induced NF-κB signaling [16]. Remarkably the standard anti-inflammatory drug dexamethasone that blocks NF-κB-induced expression of inflammatory mediators by many cell types did not inhibit thrombin-induced cytokine/chemokine production by ARPE-19, but did inhibit this in lung fibroblasts (HFL-1). Thrombin-induced *PDGFB* expression was not inhibited by dexamethasone in both ARPE-19 and HFL-1 cells. In contrast the NF-κB inhibitor SC-514 did inhibit thrombin-induced cytokine/chemokine and *PDGFB* expression by ARPE-19 and HFL-1 cells. The observed unresponsiveness of ARPE-19 to dexamethasone is most likely related to the low expression of glucocorticoid receptor, which was ~4 fold lower at the mRNA level than that observed in HFL-1. Although not examined in the current study, low expression of glucocorticoid receptor transcripts have also been described in human primary RPE cells [19]. Our data therefore suggest that dexamethasone likely is unable to inhibit powerful thrombin-induced responses by RPE cells. However RPE cells are important producers of cytokines, chemokines and growth factors that shape the inflammatory environment in PVR, which stresses the need for compounds that inhibit inflammatory mediator production by RPE cells [16, 20].

Recently we demonstrated that thrombin activity is increased in vitreous from PVR patients where it is a major factor contributing to vitreous-induced production of CCL2, CXCL8, GM-CSF and IL-6 by ARPE-19 cells. Here we demonstrate that dabigatran inhibits PVR vitreous-induced *CCL2, CXCL8, GMCSF* and *IL6* mRNA expression in ARPE-19. Therefore we consider inhibition of intravitreal thrombin activity with dabigatran as treatment option to reduce the pro-inflammatory activation of RPE cells in PVR.

Dedifferentiation of RPE cells into contractile extracellular matrix synthesizing myofibroblasts is a key event in the formation of fibrotic membranes in PVR [8, 20]. Thrombin is an important driver of this dedifferentiation process which depends on autocrine release of PDGF-BB and subsequent PDGF-receptor activation [12]. Here we found that dabigatran inhibits thrombin and PVR vitreous-induced expression of *PDGFB* mRNA in ARPE-19, suggesting that dabigatran may prevent thrombin-induced fibrotic responses in RPE cells.

Taken together our data suggest that thrombin inhibition with a DTI as dabigatran may be an interesting therapeutic option in the prevention of PVR development. It should however be taken into account that the use of a compound as dabigatran, with potential side effects including hemorrhages, should be introduced with great precaution [21-23]. Moreover in case of PVR prevention the optimal clinical window of opportunity as well as route of administration (oral vs local) remain to be determined.

Acknowledgments

The authors thank Rosanne Veerman and Pauline Arendsz for their technical support. The research for this manuscript was (in part) performed within the framework of the Erasmus Postgraduate School Molecular Medicine. The research in this study was financially supported by Combined Ophthalmic Research Rotterdam (CORR-Project code: 3.1.0) and the International Retinal Research Foundation, Birmingham, Alabama.

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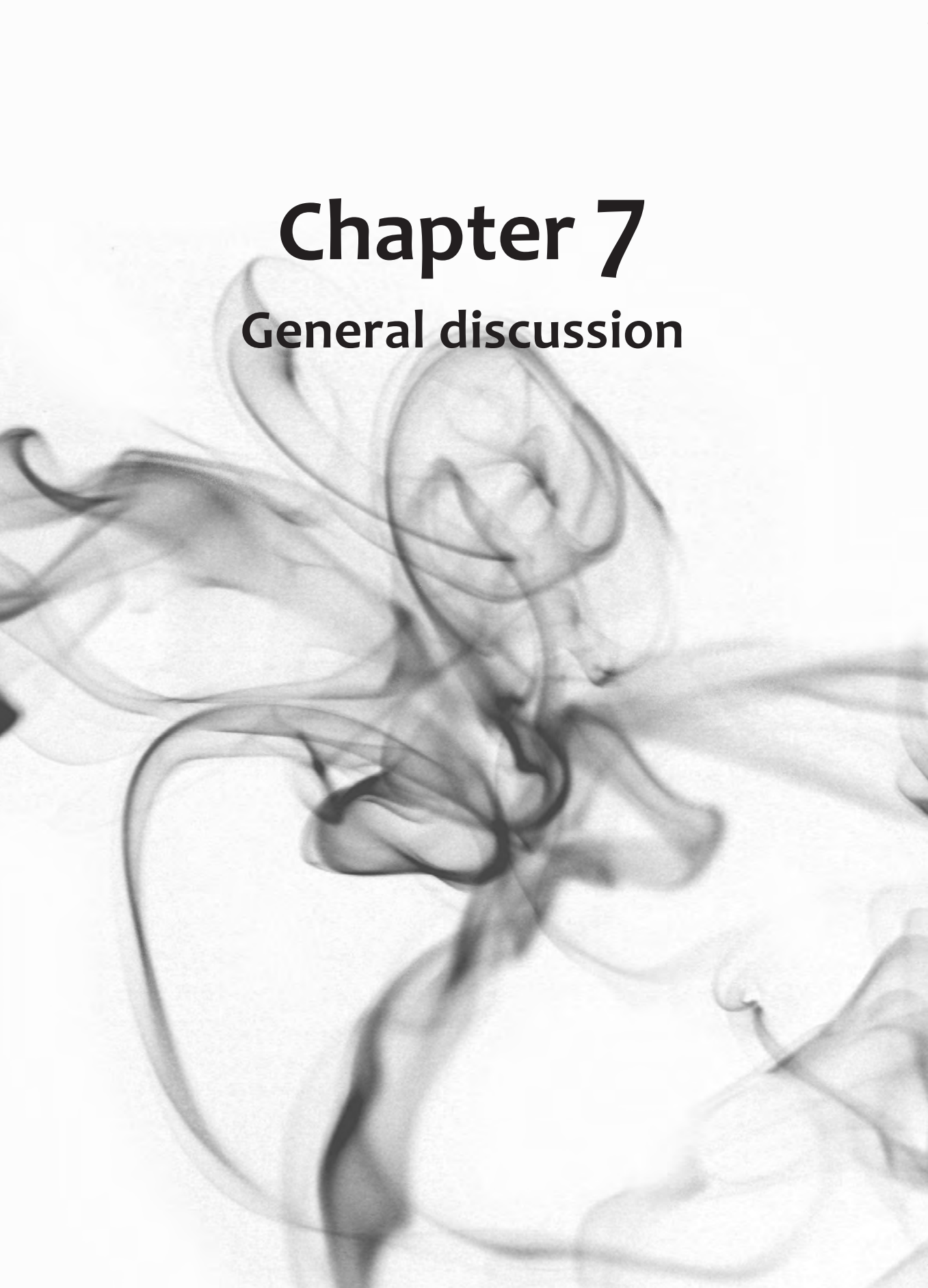
Chapter 7
General discussion



General discussion

The development of proliferative vitreoretinopathy (PVR) is characterized by inflammation followed by the formation of contractile fibroproliferative membranes which can cause the retina to detach [1, 2]. In most cases PVR is the result of an unsuccessful retinal detachment surgery in patients with a rhegmatogenous retinal detachment (RRD) [3]. Approximately 10% of the RRD patients develop PVR and have to undergo retinal detachment surgery at least once more [3]. To date the cause of PVR development remains unclear. Previous studies demonstrated that retinal pigment epithelial (RPE) cells are key players in PVR pathogenesis and that (myo)fibroblasts, glial cells and macrophages are important contributors as well [3]. Also, increased levels of platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β and vascular endothelial growth factor (VEGF) in vitreous of patients are important contributors to PVR pathogenesis, as they have been demonstrated to drive the processes which result in the formation of these contractile fibroproliferative membranes [3]. A better understanding of the processes involved in PVR development may provide better treatment options or even prevent PVR from developing.

In the eye the non-pigmented cells of the ciliary epithelium and the endothelium of the iridal capillaries constitute the blood-aqueous barrier, whereas the RPE and endothelial cells of the retinal vessels form the blood-retinal barrier (BRB) [4]. Breakdown of the BRB is part of RRD pathophysiology and subsequently serum constituents can gain access to the inner eye in higher than usual concentrations [5]. Breakdown of the BRB in patients with RRD was found to result in some degree of intra-ocular activation of the coagulation cascade [5]. By others it was demonstrated that the coagulation proteins activated factor X (FXa) and thrombin induce inflammatory and fibrotic responses in liver, kidney and lung [6-9]. Since similar processes may occur in the eye after BRB breakdown it is hypothesized in this thesis that FXa and thrombin induce PVR associated processes like cytokine and growth factor production, proliferation, and differentiation by RPE cells. The studies in this thesis used the human RPE cell line ARPE-19 but also primary human RPE cultures. The ARPE-19 cell line may display limitations as these cells may show some differences compared to primary RPE for instance on gene expression profiles, but also on biological behavior like trans-epithelial resistance development [10-12]. However, the studies performed with primary RPE generally showed similar responses to FXa and thrombin [13, 14]. Therefore the studies performed in this thesis support the hypothesis and demonstrate that FXa and thrombin can play a comprehensive role in PVR development, by inducing inflammatory and fibrotic responses by RPE cells as depicted in figure 1 [13-15].

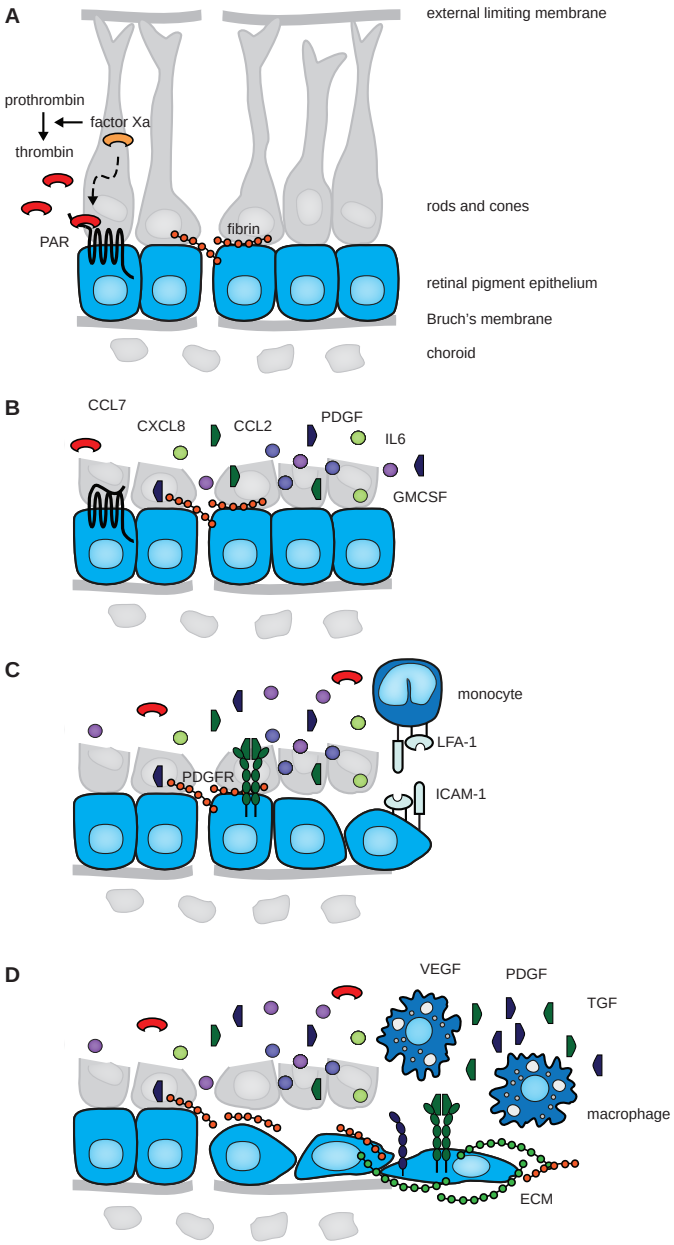


Figure 1: In PVR development the coagulation cascade is activated. The coagulation proteins FXa and thrombin induce inflammatory and fibrotic responses by RPE in a PAR-dependent manner. The inflammatory response is characterized by increased production of pro-inflammatory cytokines, chemokines and adhesion molecules. The increased cytokine/chemokine levels contribute to recruitment and activation of monocytes followed by their differentiation into growth factor producing macrophages. This latter process involves at least the adhesion molecules CD18 and ICAM-1. The induced fibrotic response includes de-differentiation of RPE into ECM and growth factor producing myofibroblasts. The combined effect of the inflammatory and fibrotic responses results in the formation of contractile fibroproliferative membranes, which represent the pathological end-stage of PVR, and can cause further retinal (re)detachment.

Breakdown of the BRB has been associated with RRD suggesting that BRB breakdown is an early event in PVR pathogenesis [5, 16-18]. However, no difference was observed in intravitreal thrombin activity between patients with RRD who don't develop PVR after surgery and patients with RRD who do develop PVR after surgery. Only the vitreous of patients with established PVR contained elevated levels of intravitreal thrombin activity, suggesting that BRB breakdown or increased BRB breakdown is a late event in PVR pathogenesis [15]. Indeed, the value of anterior chamber laser flare measurements (as a measure of BRB breakdown) to predict which patients with RRD are at risk for developing PVR, is far from clear (Mulder *et al*, ARVO 2015). BRB breakdown as late event in PVR pathogenesis may be explained by the formed fibroproliferative membranes which, due to their contractile properties, cause more severe BRB breakdown of the retinal vessels, whereas breakdown of the BRB at the level of RPE is caused by the progressive accumulation of cytokines. It can also be suggested that local production of thrombin in the retina is involved, as was demonstrated in the cornea as well as in monocytes and macrophages under pathological conditions [19-24]. Therefore it remains unclear whether RPE activation by coagulation proteins is the initiator for PVR development or a contributor to the progression of PVR development.

The presence of macrophages in PVR membranes may be indicative for their contribution to PVR pathogenesis via their capacity to produce large amounts of growth factors [3, 25-28]. Chemokines produced by the thrombin-activated RPE likely contribute to monocyte/macrophage recruitment which subsequently contribute to the inflammatory and fibrotic processes [14, 15]. However, thrombin itself has also been described to be a chemoattractant for fibroblasts, monocytes, neutrophils and RPE [29-33]. Nevertheless, it has been demonstrated before that co-culturing monocytes and RPE results in increased chemokine production and that when thrombin is added to the monocyte-RPE co-cultures, chemokine production increases [34]. In addition to these findings, the findings in Chapter 4 suggest that thrombin regulates the differentiation of monocytes into growth factor producing macrophages, which most likely involves integrin mediated cellular interaction with RPE.

How does this proposed series of events relate to the findings of numerous other studies on pathogenesis and treatment of PVR? In vitro studies and studies in animal models have suggested major roles for hepatocyte growth factor, PDGF, VEGF and TGF-β [35-43]. Analysis of patients' subretinal fluid and vitreous has shown increased concentrations of many cytokines and growth factors, and studies in patients have identified genetic polymorphisms in loci related to TNF-α, TGF signaling and apoptosis pathways as risk factors for PVR [44-50]. However, the findings in all these studies fit our hypothesis as the far majority of these described mediators and processes are regulated in RPE upon thrombin-activation. Therefore, the studies in this thesis and their subsequent target (thrombin) have the elegance of simplicity: thrombin may be an early or late, initiating or contributing molecule and is compatible with many of the downstream effects reported in other studies.

Clinical applications

To influence the development of PVR multiple steps of the process could be targeted, with a preference for interfering with initiating steps rather than later manifestations. It remains hard to be sure that the initiator can be targeted, but the studies in this thesis suggest that interference with thrombin is a feasible choice. Targeting the coagulation cascade is of particular interest because this process is an early manifestation of retinal detachment, can initiate multiple subsequent steps, and can be influenced with blocking agents which are already clinically available [5, 51, 52].

However, in five clinical trials to prevent PVR with heparin or low molecular weight heparins (LMWH) in combination with steroids or 5-fluorouracil (5-FU), there was a measurable reduction in PVR in only one [53-57]. There are several explanations for this lack of effectivity. First, for heparin and LMWH to function properly, antithrombin III (ATIII) is necessary as a catalyst to inactivate FXa and/or thrombin [58]. It remains unclear whether there is sufficient ATIII present in the vitreous for these anti-coagulants to be effective (below detection limit, unpublished data, Bastiaans *et al*). Second, heparin could only be used as a peroperative perfusion, and a single hour of anti-thrombin action is unlikely to influence a fibrotic process that occurs over a period of several weeks. A third explanation may be that the heparin (dalteparin) used in these studies, is more effective against FXa than against thrombin, the more potent inducer of fibrotic changes [59]. However, it is to be expected that FXa inhibition results in decreased thrombin generation and thus decreased PAR signaling.

For most of these reasons a direct thrombin-inhibitor would be more preferable. Dabigatran (Pradaxa) is a clinically available direct thrombin-inhibitor with hirudin-like properties [60-62]. In Chapter 6 it was demonstrated that the effects of PVR vitreous on RPE can be inhibited by dabigatran. These findings make the use of dabigatran an interesting therapeutic strategy for treatment or prevention of PVR development.

However, two important issues need to be addressed:

1. The use of anticoagulants needs to be considered carefully, since side effects like hemorrhages in the brain and other organs have been reported [63]. Systemic administration of anticoagulants like dabigatran to all patients at risk for PVR development may expose many patients to these side effects. It would be desirable to be able to select patients at high risk for PVR only. A reliable biomarker for this purpose remains to be found. But the data from Chapter 5 suggest that CCL4 may represent such a biomarker since this was found elevated in vitreous of patients who developed PVR 6 months after vitrectomy, compared to those patients who did not develop PVR after vitrectomy. Therefore more research regarding the role of CCL4 in PVR development may be of interest.
2. Thrombin has a relatively short half-life. In normal human plasma the half-life of thrombin is ~56.4 ± 4.7 seconds. In antithrombin-deficient plasma the half-life of thrombin is ~168.2 ± 14.9 seconds [64]. Since the duration of BRB breakdown is at least three weeks, patients should be treated during that period [65]. The same arguments would be applicable for therapeutically targeting PARs or tyrosine-kinase inhibitors to prevent them from signaling.

Various intravitreal proteins have been suggested to contribute to PVR development. These proteins and the processes they regulate implicate that PVR pathogenesis comprises more than one variable. Therefore a multivariable therapeutic approach may be most favourable. The studies in this thesis demonstrate that many of the processes involved in PVR pathogenesis, including the upregulated expression of cytokines and

growth factors, are downstream of PAR signaling [13-15]. PARs cannot only be activated by thrombin but by many other proteases as well, of which numerous have been found in vitreous of patients with vitreoretinal disorders. [66, 67]. Studies in this thesis and others also clearly demonstrate an important role for PDGF-receptor signaling [13, 41, 68]. The elevated levels of different PDGF isoforms induce fibrotic responses via PDGFR-α and PDGFR-β activation [68-71]. In addition, VEGF and FGF have been described to activate PDGFR-α through transactivation via their own receptors [72, 73]. In fibroproliferative membranes from patients with PVR mostly PDGFR-α was found activated [71]. Therefore, combined targeting of receptors like PAR and PDGFR may be even more efficient in preventing PVR development. Vorapaxar (Zontivity) is a new and clinically available PAR1 inhibitor which has so far showed limited side effects [74-76]. Tyrosine-kinase inhibitors like imatinib (Glivec), dasatinib (Sprycel) and nintedanib (Vargatef) may be efficient PDGFR blockers which are already clinically available. The last one (nintedanib) also blocks VEGF-receptors and FGF-receptors [77-79].

The role of coagulation proteins in other vitreoretinal disorders

The fibrotic processes induced by thrombin do not only occur in PVR development, but in other vitreoretinal disorders like exudative age-related macular degeneration (ARMD) and proliferative diabetic retinopathy (PDR) as well [80-83]. Components of the coagulation cascade have been found in vitreous of these patients as well and as such it is likely that in these disorders thrombin also contributes to pathogenesis [84-88].

The exudative or wet form of ARMD is associated with severe vision loss that can occur rapidly [89]. This form of ARMD is characterized by choroidal neovascularization, stimulated by VEGF [89,90]. These newly formed vessels leak blood and fluid, which can result in coagulant protein-driven activation of PARs expressed on the various cell types in the retina. Studies in histological specimens from both humans and animals have demonstrated the presence of macrophages as the main infiltrating inflammatory cells in ARMD lesions, particularly in the choroidal neovascular membranes (CNVM) [91]. The precise role of macrophages in ARMD is still unclear. However, it can be expected that they exert similar effects as in PVR membranes. This is supported by the finding that the macrophages in the CNMV are capable of expressing pro-angiogenic factors such as VEGF and pro-inflammatory molecules, as in PVR [92].

The vitreous of patients with PDR contains elevated levels of prothrombin and ATIII compared to patients without diabetes mellitus and diabetes with no apparent diabetic retinopathy [93]. PDR is characterized by retinal neovascularization resulting in increased retinal haemorrhages and increased levels of VEGF in the vitreous. These processes result in the formation of fibrovascular membranes. In these membranes endothelial cells and macrophages are prominently present [94, 95]. Also, these fibrovascular membranes can cause retinal detachment by contraction.

Collectively, both ARMD and PDR are characterized by increased levels of locally produced and serum-derived proteins like VEGF in the vitreous, membrane formation and macrophage accumulation causing choroidal (ARMD) or retinal (PDR) neovascularization. In ARMD and PDR, similar to PVR development, macrophage accumulation and membrane formation are part of fibrovascular tissue. Apart from the mainstay of treatment: anti-VEGF, inhibition of thrombin may therefore be of some benefit in these patients as well, possibly to modify the fibrotic part of the fibrovascular process.

A role for other coagulation proteins in PVR development

The coagulation proteins FXa and thrombin have widely been discussed for their activating properties. However, other components from the coagulation cascade, including fibrinogen, fibrinopeptides and fibrin fragments, have been described to induce cellular responses as well. In bronchial epithelial cells fibrinogen has been described to induce CCL2, CCL3 and CCL4 production by macrophages in a Toll-like receptor (TLR)4-dependent manner and fibrinopeptides to induce IL-5 and IL-13Rα1 expression in a TLR4-dependent manner [96, 97]. Fibrinopeptides are side products generated by the cleavage of fibrinogen by thrombin. These side products consist of 16 amino acids long fibrinogen α chains and 14 amino acids long fibrinogen β chains [98]. RPE cells also express TLR4 which suggests that formed fibrinopeptides, as the result of intra-ocular activation of the coagulation cascade, may induce TLR4-mediated activation of RPE. Also activated protein C, a natural anticoagulant, has been described to induce cellular responses which are associated with neovascularization and wound healing in a PAR-dependent manner [99-102].

During fibrinolysis, a counter regulatory mechanism for activation of the coagulation cascade, fibrin is cleaved by plasmin, resulting in clot lysis and an accompanied generation of fibrin fragments such as D and E fragments [103]. These fragments are chemotactic for leukocytes, but can also induce leukocyte migration by induction of cytokine/chemokine production [103]. Other components of the fibrinolytic system like protease-activated receptor-1 and the plasmin-a_2-antiplasmin-complex have been found elevated in vitreous of patients with PVR as well [16]. However, whether these components affect the earlier described inflammatory and fibrotic properties of coagulation proteins remains to be investigated.

Collectively these findings suggests additional roles for other components of the coagulation cascade in the activation and continuation of inflammatory and fibrotic responses by RPE which have not been investigated so far. Some of these interactions, though, may have occurred in the experiments with vitreous of patients. As such, dampening thrombin action continues to be a plausible approach.

Concluding remarks

The development of PVR remains the major cause of unsuccessful retinal detachment surgery. Vitreoretinal surgeons have managed to improve their surgical skills and are relatively successful in reattaching the retina, with, however, unfortunately very modest functional results. No improvement has been made with anti-proliferative agents, steroids and heparin to prevent or treat PVR. The use of new direct thrombin-inhibitors, possibly in combination with new and more efficient receptor tyrosine-kinase inhibitors to target growth factor effectors, or even more speculatively antibodies to individual growth factors, cytokines or receptors may contribute to a prevention treatment for PVR development.

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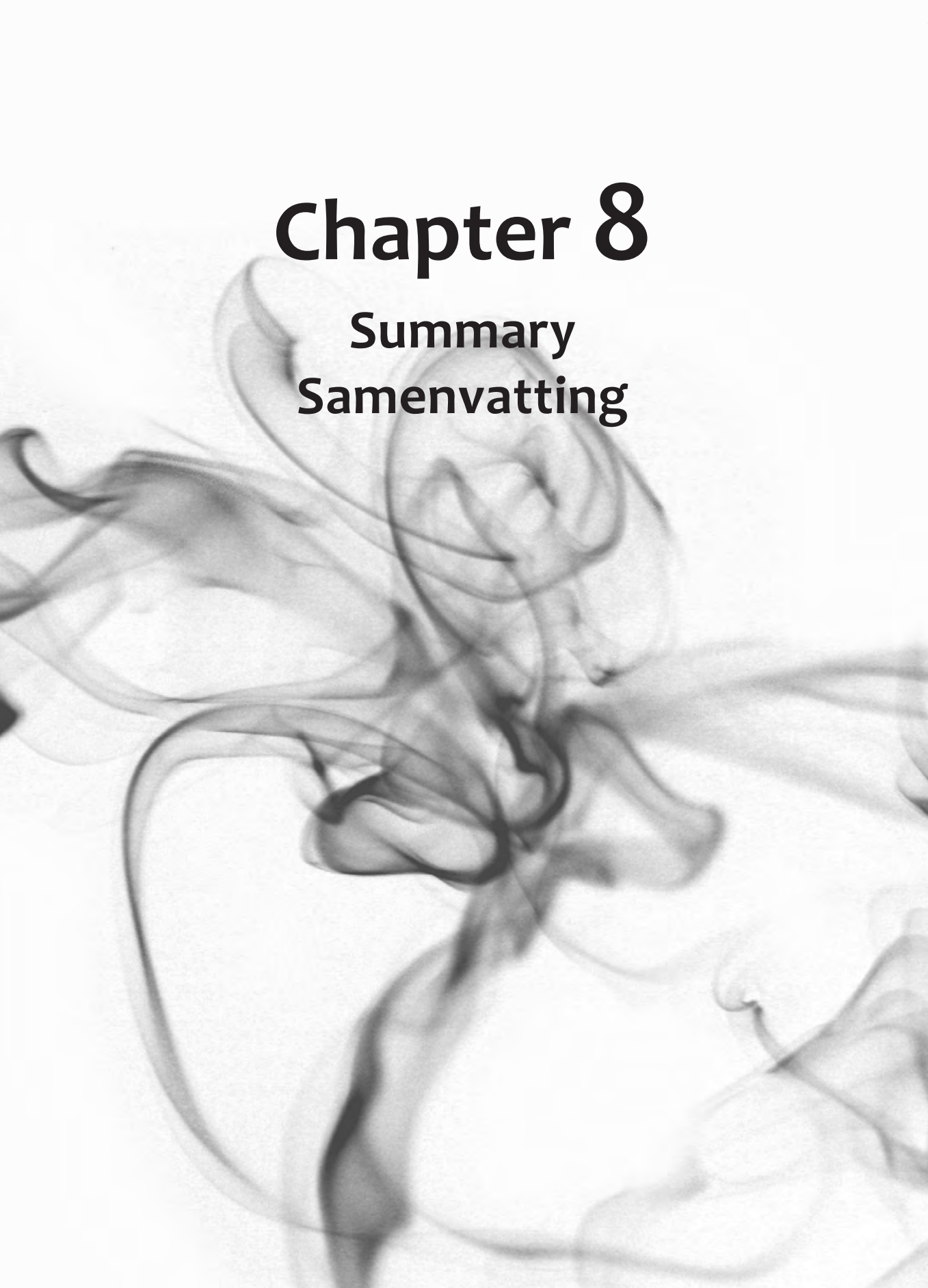
Chapter 8

Summary
Samenvatting



Summary

The pathogenesis of proliferative vitreoretinopathy (PVR) comprises inflammatory and fibrotic processes which result in the formation of fibrotic epiretinal, intraretinal and/or subretinal membranes. These membranes can cause the retina to detach with resultant loss of vision. PVR develops in approximately 10% of the patients with a rhegmatogenous retinal detachment or can develop as a result of trauma. To date, surgery is the only treatment option for PVR, but this is not always successful. A better understanding of the molecular and cellular processes involved in the pathogenesis of PVR may lead to better treatment options.

The role for coagulation proteins in inflammatory and fibrotic responses in organs including the lungs and kidneys is well established. Here, they stimulate proliferation and the production of cytokines and extracellular matrix molecules by several cell types, including (myo)fibroblasts. PVR development is associated with breakdown of the blood-retinal barrier, which results in intraocular activation of the coagulation cascade and subsequent exposure of retinal pigment epithelial (RPE) cells to coagulation proteins. RPE cell activation is considered to represent a central event in the development of PVR. Activated RPE cells can produce chemokines and cytokines, involved in immune cell recruitment, activation and differentiation. They produce extracellular matrix proteins, including collagen, and are abundantly present in fibroproliferative membranes. In contrast to what is known about the role for coagulation proteins in inflammatory and fibrotic responses in organs such as the lungs and kidneys, the contribution of coagulation proteins to RPE cell activation in PVR is still poorly understood. In this thesis it was hypothesized that the coagulation proteins activated factor X (FXa) and thrombin contribute to PVR development via the induction of pro-inflammatory and pro-fibrotic processes in RPE cells. These coagulation proteins may thus represent an attractive therapeutic target to prevent or treat PVR.

Chapter 2 of this thesis describes that FXa and thrombin stimulate the production of several cytokines, chemokines and growth factors by RPE cells. Many of these (e.g. IL-6, CCL2 and PDGF) have previously been linked to inflammation and fibrosis in PVR. Both FXa and thrombin established this effect via protease-activated receptor-1 induced activation of the NF-κB signaling pathway. Remarkably, differences existed between regulation of the production of the cytokines, chemokines and growth factors by FXa and thrombin in the RPE cells. It was found that thrombin generally appeared to exert stronger stimulatory effects than FXa.

Chapter 3 demonstrates that thrombin induces de-differentiation of RPE cells into myofibroblasts, expressing α-smooth muscle actin and producing collagen. This is associated with loss of typical epithelial characteristics such as expression of the tight junction protein zonula occludens-1. In addition, the data in this chapter demonstrate that this thrombin induced process of epithelial-mesenchymal transition of RPE cells into myofibroblasts, involved the induction of autocrine PDGFR signaling.

Chapter 4 shows that thrombin enhances monocyte-to-macrophage differentiation in monocyte-RPE co-cultures. This differentiation process most likely depends on direct physical interaction between monocytes/macrophages and RPE cells and involves at least the adhesion molecules CD18 and ICAM-1. The thrombin enhanced monocyte-to-macrophage differentiation resulted in increased production of the pro-fibrotic mediators PDGF-BB, TGF-β_2 and VEGF-A, which may suggest that the newly formed macrophages obtained an M2-phenotype.

Chapter 5 demonstrates that vitreous of patients with established PVR contains elevated thrombin-activity, in contrast to vitreous of patients with rhegmatogenous retinal detachment, macular puckers or macular holes. Moreover, this enhanced thrombin-

activity in PVR vitreous was found to represent a major activator of pro-inflammatory and pro-fibrotic pathways in RPE cells.
Altogether the data presented in **Chapter 2-5** implicate a comprehensive role for especially thrombin in the pathogenesis of PVR. Thrombin may thus represent an attractive therapeutic target to treat or prevent PVR from developing, especially since direct thrombin inhibitors are clinically available. **Chapter 6** describes the effects of the clinically available direct thrombin inhibitor dabigatran (Pradaxa) on thrombin and PVR-vitreous-induced activation of RPE cells. The effect of dabigatran was compared to that of dexamethasone. Dabigatran efficiently blocked thrombin-induced production of cytokines, chemokines and growth factors by RPE cells, while dexamethasone largely failed to do so. This latter may be related to low glucocorticoid receptor expression in RPE cells. Importantly, dabigatran also inhibited cytokine, chemokine and growth factor production by RPE cells induced by (thrombin containing) PVR-vitreous, indicating that dabigatran is able to block thrombin-activity within the vitreal matrix.
In conclusion, the studies described in this thesis provide important new insight into the contribution of especially thrombin to the induction of pro-inflammatory and pro-fibrotic events in RPE cells during PVR pathogenesis. Inhibition of intravitreal thrombin-activity may thus represent an attractive therapeutic option to treat or prevent PVR development, and future studies into this are therefore warranted.

Samenvatting

De ontwikkeling van proliferatieve vitreoretinopathie (PVR) wordt gekenmerkt door inflammatoire en fibrotische processen die uiteindelijk resulteren in de ontwikkeling van fibroproliferatieve membranen. Deze membranen kunnen er voor zorgen dat het netvlies loslaat wat vervolgens kan resulteren in zichtvermindering. PVR ontwikkelt zich bij ongeveer 10% van de patiënten die zijn geopereerd i.v.m. een rhegmatogene netvliesloslating of als gevolg van trauma. Tot de dag van vandaag is een nieuwe operatie nog altijd de enige optie om PVR te behandelen. Een beter inzicht in de moleculaire en cellulaire processen die plaatsvinden zowel voor als tijdens de ontwikkeling van PVR, zou kunnen resulteren in betere behandelingsmogelijkheden.
De rol van stollingseiwitten in inflammatoire en fibrotische processen in organen als de long en lever is breed erkend. In deze organen stimuleren stollingseiwitten proliferatie en de productie van cytokinen en extracellulaire matrixmoleculen in verschillende celtypen, waaronder (myo)fibroblasten. De ontwikkeling van PVR is geassocieerd met schade aan de bloed-retina barrière, gevolgd door intra-oculaire activatie van de stollingscascade. Dit resulteert vervolgens in blootstelling van retinaal pigment epitheel (RPE) cellen aan stollingseiwitten. Van RPE cellen, die zich tussen de fotoreceptoren en het choroïd bevinden, wordt aangenomen dat ze een belangrijke rol spelen in PVR ontwikkeling, omdat ze dominant aanwezig zijn in PVR membranen. Bovendien produceren ze na activatie cytokinen, chemokinen en groeifactoren die bijdragen aan (myo)fibroblast-differentiatie, de productie van extracellulaire matrixmoleculen en activatie en rekrutering van immuuncellen. In tegenstelling tot de rol van stollingseiwitten in inflammatoire en fibrotische processen in organen als de long en lever, is nog maar weinig bekend over de bijdrage van stollingseiwitten aan RPE activatie bij PVR ontwikkeling. In dit proefschrift is gehypothetiseerd dat de stollingseiwitten geactiveerde factor X (FXa) en trombine bijdragen aan PVR ontwikkeling via inflammatoire en fibrotische processen in RPE. Deze stollingseiwitten zouden daardoor mogelijk interessante therapeutische targets zijn voor de voorkoming of behandeling van PVR.
Hoofdstuk 2 van dit proefschrift beschrijft dat FXa en trombine de productie van diverse cytokinen, chemokinen en groeifactoren door RPE stimuleren. Deze cytokinen, chemokinen en groeifactoren (zoals IL-6, CCL2 en PDGF) zijn al vaker geassocieerd met inflammatoire en fibrotische processen in PVR ontwikkeling. Het effect van zowel FXa als trombine verloopt via protease-geactiveerde receptor-1 geïnduceerde activatie van de NF-κB signalerings-pathway. Opvallend is dat er verschillen zijn tussen FXa en trombine in regulatie van de productie van cytokinen, chemokinen en groeifactoren door RPE. In deze studie bleek trombine de meest potente regulator te zijn van de productie van cytokinen, chemokinen en groeifactoren.
Hoofdstuk 3 laat zien dat trombine de-differentiatie induceert van RPE cellen tot (myo) fibroblasten die verhoogd α-smooth muscle actine tot expressie brengen en collageen produceren. Dit proces is geassocieerd met verlies van epitheliale karaktereigenschappen zoals de expressie van het tight junction eiwit zonula occudens-1. Daarnaast laat de data in dit hoofdstuk zien dat de door trombine geïnduceerde de-differentiatie van RPE cellen gereguleerd wordt via autocrine PDGFR-signalering.
Hoofdstuk 4 beschrijft dat trombine de differentiatie van monocyten tot macrofagen in monocyt-RPE co-kweken versterkt. Dit differentiatieproces lijkt afhankelijk te zijn van een directe interactie tussen de monocyten/macrofagen en de RPE cellen, waarbij in ieder geval de adhesiemoleculen CD18 en ICAM-1 betrokken zijn. Deze door trombine versterkte macrofaagdifferentiatie resulteert in verhoogde productie van pro-fibrotische mediatoren als PDGF-BB, TGF-β_2 en VEGF-A. Dit suggereert dat de gevormde macrofagen mogelijk fibrotische M2-macrofagen zijn.

Hoofdstuk 5 demonstreert dat het glasvocht van patiënten met PVR een verhoogde hoeveelheid aan trombine-activiteit bevat. Dit is niet het geval voor glasvocht van patiënten met macula gaten, macula puckers en rhegmatogene netvliesloslatingen. De verhoogde trombine-activiteit in PVR-glasvocht is een sterke activator van pro-inflammatoire en pro-fibrotische routes in RPE cellen.
Alles bij elkaar laat de data in **Hoofdstuk 2 t/m 5** zien dat er een belangrijke rol is voor trombine in PVR ontwikkeling. Om ontwikkeling van PVR te voorkomen, zou trombine dan ook een therapeutische target van interesse kunnen zijn, temeer omdat er directe trombine remmers klinisch beschikbaar zijn.
Hoofdstuk 6 beschrijft de effecten van de klinisch beschikbare directe trombine remmer dabigatran (Pradaxa) op door trombine en PVR-glasvocht geïnduceerde activatie van RPE cellen. Dit effect van dabigatran is vergeleken met dat van dexamethason. Dabigatran remt de door trombine geïnduceerde productie van cytokinen, chemokinen en groeifactoren door RPE, in tegenstelling tot dexamethason. Dit zou kunnen worden verklaard door de relatief lage expressie van de glucocorticoïd receptor in RPE cellen. Belangrijk is dat dabigatran ook door PVR-glasvocht (met verhoogde trombine activiteit) geïnduceerde productie van cytokinen, chemokinen en groeifactoren door RPE remt. Dit geeft de indicatie dat dabigatran intravitreale trombine activiteit kan remmen in een glasvochtmatrix.
In conclusie voorzien de studies beschreven in dit proefschrift in belangrijk nieuw inzicht in de bijdrage van vooral trombine bij de inductie van inflammatoire en fibrotische processen door RPE cellen tijdens de ontwikkeling van PVR. Inhibitie van intravitreale trombine activiteit zou daarom mogelijk een interessante therapeutische strategie zijn om de ontwikkeling van PVR te voorkomen of te behandelen. Verdere studies naar dergelijke behandelmethoden zijn daarom gewenst.

8

Chapter 9
Appendices

Abbreviations
Dankwoord

Abbreviations

6Ckine	Secondary lymphoid tissue chemokine/CCL21
APC	Allophycocyanin/antigen presenting cell
APC-EPCR	Activated protein C – endothelial (activated) protein C receptor
AR	Androgen receptor
ARMD	Age-related macular degeneration
ATIII	Antithrombin III
Axl	AXL receptor tyrosine kinase
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BLC	B-lymphocyte chemoattractant/CXCL13
BMP	Bone morphogenic protein
BRB	Blood-retinal barrier
BSA	Bovine serum albumin
BTC	Betacellulin
CCL	Chemokine (C-C-motif) ligand
CCR	Chemokine (C-C-motif) receptor
CD	Cluster of differentiation
cDNA	Copy deoxyribonucleic acid
CRALBP	Cellular retinaldehyde-binding protein
Ct	Cycle threshold
CTACK	Cutaneous T-cell-attracting chemokine/CCL27
CTGF	Connective tissue growth factor
CXCL	Chemokine (C-X-C-motif) ligand
CXCR	Chemokine (C-X-C-motif) receptor
DAPI	4',6-diamidino-2-phenylindole
DMEM/F-12	Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12
DTI	Direct thrombin inhibitor
ECM	Extracellular matrix
EDTA	Ethylenediamine tetra-acetate
EGF	Epidermal growth factor
ELISA	Enzyme-enzyme linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
ENA-78	Epithelial-derived neutrophil-activating protein 78/CXCL5
FACS	Fluorescence activated cell sorter
FCP	Fibrinogen cleavage product
FCS	Fetal calf serum
FII (a)	(activated) factor II/(pro)thrombin
FITC	Fluorescein isothiocyanate
FX(a)	(activated) factor X
GCP-2	Granulocyte chemotactic protein 2/CXCL6
GDF	Growth differentiation factor
GDNF	Glial cell derived neurotrophic factor
GH	Growth hormone
GM-CSF	Granulocyte macrophage colony-stimulating factor
GRO	Growth-regulated alpha protein/CXCL1

HAT	Transmembrane protease serine 11D
HB-EGF	Heparin-binding EGF-like growth factor
HCC	Thyroid carcinoma, hurthle cell
HGF	Hepatocyte growth factor
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
I-309	Inflammatory cytokine-309/CCL1
ICAM	Intercellular adhesion molecule
IFN-γ	Interferon gamma
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IKK2	Nuclear factor kappa-B kinase subunit beta
IL	Interleukin
I-TAC	Interferon-inducible T-cell alpha chemoattractant/CXCL11
KLK	Kallikrein
LDH	Lactate dehydrogenase
LIF	Leukemia inhibitory factor
LIGHT	Tumor necrosis factor (ligand) superfamily, member 14
MAC-1	Macrophage-1 antigen
MASP-1	Mannon binding lectin serine peptidase 1
MCP	Monocyte chemotactic protein
M-CSF	Macrophage colony-stimulating factor
MDC	Macrophage-derived chemokine/CCL22
MDM2	Mouse double minute 2 homolog
MET	Mesenchymal-to-epithelial transition
MIF	Macrophage migration inhibitory factor
MIG	Monokine induced by gamma interferon
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MPIF-1	Myeloid progenitor inhibitory factor 1/CCL23
mRNA	Messenger ribonucleic acid
MSP	Macrophage-stimulating Protein
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAP-2	Napsin B aspartic peptidase
NF-kB	Nuclear factor-kappa B
NT-3	Neurotrophin 3
OD	Optical density
OPG	Osteoprotegerin
OPN	Osteopontin
PAI	Plasminogen activator inhibitor
PAR	Proteinase-activated receptor
PARC	Pulmonary and activation-regulated chemokine/CCl18
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PDGF	Platelet derived growth factor
PDGF-R	Platelet derived growth factor receptor
PDR	Proliferative diabetic retinopathy

PE	Phycoerythrin
PEDF	Pigment epithelial derived growth factor
Pen C 13	Penicillium citrinum 13
PerCP	Peridinin chlorophyl protein
PF4	Platelet factor 4
PIGF	Phosphatidylinositol glycan anchor biosynthesis, Class F
PMA	Phorbol-12-myristate-13-acetate
POS	Photoreceptor outer membrane segments
PPV	Pars plana vitrectomy
PTX3	Pentraxin 3
PVR	Proliferative vitreoretinopathy
RANKL	Receptor activator of nuclear factor kappa-B ligand
RANTES	Regulated on activation, normal T-cell expressed and secreted/CCL5
RPE	Retinal pigment epithelium
RPMI-1640	Roswell park memorial institute-1640 medium
RQ-PCR	Real-time quantitative polymerase chain reaction
RRD	Rhegmatogenous retinal detachment
RVO	Retinal vein occlusion
SCF	Stem cell factor
SDF-1a	Stromal cell-derived factor-1A/CXCL12
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophorese
SEM	Standard error of the mean
SMAD7	Mothers against decapentaplegic 7
TARC	Thymus and activation-regulated chemokine/CCL17
TBS	Tris-buffered saline
TECK	Thymus expressed chemokine/CCL25
TF	Tissue factor
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TJP1	Tight junction protein-1
TLR	Toll-like receptor
TMPRSS2	Transmembrane protease serine 2
TNF	Tumor necrosis factor
TP53	Tumor protein 53
tPA	Tissue plasminogen activator
TRAIL	TNF-related apoptosis inducing ligand
TSLP	Thymic stromal lymphopoietin
uPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
ZO-1	Zonula occludens-1
α-SMA	alpha-smooth muscle actin
β-NGF	beta-nerve growth factor

Dankwoord

Wie had tot een paar jaar geleden ooit nog gedacht dat ik het dankwoord van mijn proefschrift zou schrijven? En toch is het inmiddels zover. Zo zie je maar waar een hoop inspirerende en motiverende mensen om je heen toe kunnen leiden. Ik zal dan denk ik ook wel één van de laatste zijn die zal beweren dit alles alleen voor elkaar te hebben gekregen. Tijdens dit promotietraject waren er hoge pieken maar ook diepe dalen, en dit alles heb ik kunnen delen met geweldige vrienden en collega's. Het succesvol afronden van mijn promotie heb ik te danken aan een hoop mensen, en omdat het altijd de vraag is waar te beginnen, begin ik maar gewoon in chronologische volgorde.

Dan mag ik meteen beginnen bij een zeer gewaardeerde onderzoeker binnen de afdeling Immunologie. Beste Dr. van den Burg, beste Mirjam, bij jou had ik initieel gesolliciteerd voor een positie als analist in de PID werkgroep. Al vrij snel werd voor ons beiden duidelijk dat de hoeveelheid diagnostiek die bij deze functie hoorde, niet voor mij was weggelegd. Desalniettemin heb je mijn C.V. bewaard en deze later doorgegeven aan Wim, bij wie een plek voor een research analist vrij kwam. Als jij dat destijds niet had gedaan, had ik hier nu niet gestaan. Heel erg bedankt hiervoor.

Beste Dr. Dik, beste Wim, jij gaf me de gelegenheid om me als analist verder te ontwikkelen binnen jouw groep, en later gaf je me zelfs de kans om aan dit promotietraject te beginnen; een beslissing die een risico voor ons beiden met zich meebracht. Bedankt dat je me deze keuze hebt gegeven. Daarnaast wil ik je ook zeker bedanken voor de geboden kansen waarbij ik mezelf nog verder en breder kon ontwikkelen, waaronder de mogelijkheid om relatief veel (inter)nationale congressen te bezoeken. Voor mij waren dit belangrijke momenten waar ik vaak het meeste leerde. Hierop aansluitend wil ik ook graag Prof. dr. Hooijkaas bedanken voor de mogelijkheid om aan dit promotietraject te beginnen. Beste Herbert, bedankt voor de goede zorgen. Ik wens je het allerbeste voor de toekomst.

Beste Prof. dr. van Meurs, beste Jan, u ook bedankt voor het vertrouwen en de mogelijkheid voor mij om aan dit promotietraject te mogen beginnen. Onze korte overleggen waren voor mij altijd erg motiverend. Het feit dat u wat verder van het laboratorium af staat, was voor mij een goede stimulans om mijn onderzoek binnen de oogheelkunde op een andere manier te leren uitleggen, zodat het ook begrijpelijk wordt voor mensen die niet op een laboratorium werkzaam zijn.

Beste Prof. dr. van Hagen, beste Martin, ook jou wil ik graag bedanken voor het vertrouwen en je bijstand met raad en daad. Jouw wijsheid is van unieke waarde. Ook onder jouw vleugels heb ik me breder mogen en kunnen ontwikkelen; eerst op het FcRn-project met Kiki en later binnen mijn eigen promotietraject.

Beste Prof. dr. Vingerling, Prof. dr. Hofland en Prof. dr. Kijlstra bedankt dat jullie plaats wilden nemen in de kleine commissie ter beoordeling van mijn concept proefschrift. Verder ook dank aan de overige leden van de grote commissie voor jullie bijdrage.

Beste Verena, als promovendi binnen hetzelfde project waren we deels op elkaar aangewezen, ook al was het mijn taak een 'laboratory-based' rationale te vinden en was het jouw taak om die rationale te gebruiken voor het vervolg van jouw studies, waaronder de trials. Ondanks dat er soms maar weinig van het hele invries- en opslagsysteem klopte, waardeerde ik onze discussies bij de -80°C vriezer zeer. Ik wens je nog heel veel succes bij het afronden van jouw studies.

Ook veel dank aan de collega's van de afdeling Oogheelkunde voor jullie bijdrage. In het bijzonder Joeri en Emine, het door jullie verzamelde patiëntenmateriaal is van grote waarde geweest.
Kim en Leendert, jullie hebben waarschijnlijk geen idee hoe dankbaar ik jullie ben. Als kantoorgenoten hebben we heel veel gedeeld (waarvan een groot deel in snoeppotten zat) en vooral veel gelachen. Toen ik de kans kreeg om aan dit promotietraject te beginnen, waren jullie voor mij van onschatbare waarde. Ik kon me geen betere rolmodellen voorstellen. Hard werkend, flexibel en succesvol. Als ik bij jullie (en deels ook Marten en Jan Willem) de kunst van het promoveren niet had kunnen afkijken, weet ik niet of alles zo was gelopen zoals het gedaan heeft.
My sweet Asian invader Prayer. We shared the same office for the last three years. Together we were the odd ones out at the department of Immunology, since actual immunology was missing in both our projects. I take a deep bow for your endurance with all the Thai jokes we made, and I have deep respect for your perseverance to come to Holland for your PhD. The cultural and climate differences must have had a huge impact on you. Your grown (and enforced) admiration for B-Brave was maybe a bit too much, but for you we accepted it. I wish you all the best in your future career and life. Rock the Boat!
Then my other sweet Asian invader Rina. Also for you I have so much respect. It takes a lot of dedication to leave your sons and husband behind (for limited periods) to do your studies in our lab. Luckily there is Skype, so you all could stay in touch. Thank you so much for your support. For me it was of great help to have an ophthalmologist so close by. Also to you, I wish all the best in your future career and life. Gangnam on!!!
Lieve Hanna, wat heb ik toch veel aan jou gehad. Bij elkaars 'crisis' momenten naar Doppio rennen om daar alles te relativeren en tot rust te komen. Gelukkig voor ons waren die er niet zo veel en hadden we het vooral vaak heel gezellig. Je bent een topper!
Jorn, toen jij als AIO op het lab kwam, was het aantal mannelijke AIO's behoorlijk beperkt en waren we op dat vlak op elkaar aangewezen. Gelukkig ging dat meer dan prima en hebben we er een mooie tijd van gemaakt. Samen in de FC (alhoewel, hoe FC ben je als je tijdens de labdag in Thailand op het strand ligt?), organisatie van WK voetbal en Tour de France poules, of een lekker 'bakkie' in Den Haag buiten werktijd. Het resultaat van mijn waardering hiervoor pronkt nog altijd in A0 formaat op de 12de verdieping. Ik wens je heel veel succes met alles wat nog gaat komen. Jij gaat het maken!
Ook een woord van dank naar alle andere AIO's binnen de afdeling Immunologie. Britt(y), wat heb je een heerlijke humor. Ik zal ons hoekje voor 'special people' op de NVvI missen. Gelukkig hebben we ons motto nog: oog om oog, tand(vlees) om tand(vlees). En gewoon omdat het kan, nog één keertje in Koos Alberts stijl van boven naar beneden: oog, tandvlees, nier en 'iets met langerhanscellen'. Als we het dan toch over humor hebben dan kan ik H.K.H. Martine natuurlijk niet vergeten. Zonder in te veel details te treden over alle momenten waarop we hebben zitten gieren: KLAP! Fabi, my friend, never have I met someone with such a drive for a huge career in medicine and science as you. Luckily, you also know how to enjoy life. So keep rocking it! Chris, Mati and Karin, although you have left the department a while ago, I also want to thank you for some really great moments. Verder ook dank aan alle andere AIO's waarmee ik de afgelopen 4 jaar heb mogen delen: Alice, Anna, Armanda, Chris, Christina, Diana, Dew, Ewout, Ferry, Hessel, Iris, Jenna, Joey, Jojanneke, Karim, Karin, Kiki, Lieke, Lisa, Lizenka, Lucy, Maarten, Magda B, Magda R, Malou, Marieke, Marjolein, Marlieke, Naomi, Nicole,

Odilia, Prisca, Sandra, Simar, Wendy, Wida, Willem Jan, Wouter B, Wouter vdB en Zana. Ik wens jullie allen het allerbeste!

Dan is het toch echt tijd voor de twee grootste toppers van het lab: (Puzzz) Nicole en (tante) Conny. Wat had ik zonder jullie gemoeten en wat ben ik jullie ontzettend dankbaar!!! Niet alleen voor jullie keiharde werk, inzet en flexibiliteit, maar ook om wie jullie zijn. Zonder jullie had dit proefschrift er een stukje dunner uitgezien, en waren de afgelopen jaren vast en zeker veel moeizamer verlopen. Of het nou ging om (on)gein uithalen, in canon het kuikentjes lied zingen in de LAF-kasten, het jaarlijks herhalen van 1 april grappen, studenten ondervragen CSI-stijl, 'Nederlands' leren aan onze buitenlandse collega's of de loze conversaties. Alles kon en alles mocht. Het meest bijzondere vond ik nog wel dat jullie bij een half woord al voor me klaar stonden. Dan pas ga je beseffen wat collegialiteit, flexibiliteit en inzet echt inhouden. Weet dat ik het oprecht waardeer! Het kan natuurlijk ook niet anders dan dat ik met jullie aan mijn zijde mijn promotietraject wil afsluiten. Bedankt dat jullie mijn paranimfen willen zijn.

En dan de rest van het warme nestje waarin ik belandde: de IRD werkgroep. In geen enkele werkgroep zijn diagnostiek en research zo strikt gescheiden als bij ons, en toch wisten we elkaar te vinden. Marja en Marion, onze labmoeders en dynamisch duo. Jullie ook ontzettend bedankt voor jullie hulp en inzet. Ik waardeer het zeer dat jullie telkens maar weer tijd wilden inruimen voor mijn multiplex analyses. Verder wil ik uiteraard ook Benjamin, Corrien, Dagmar, Dicky, Gemma, Marten, Nicoline en Jan Willem bedanken voor de gezelligheid en talloze mooie momenten op het lab. De studenten die hebben bijgedragen aan dit project: Nada, Pauline, Peter en Rosanne, jullie ook heel erg bedankt voor jullie inzet en bijdrage. En ook de andere studenten in onze werkgroep Irma, Judith, Jacolien, Kees, Marloes, Simon, Vilma en alle anderen, bedankt voor de gezelligheid.

Gellof, Patricia, Maaike en Barbara, ook jullie wil ik heel graag bedanken voor de vele zeer mooie momenten. Wat zal ik die gaan missen. Of het nou ging om borrels, feestjes, festiviteiten buiten werktijd of al het andere wat met gezelligheid te maken heeft, we wisten elkaar te vinden. Op het moment dat het even niet zo goed ging, waren jullie er ook en dat is mij meer dan goud waard! En lieve Patries, intern ook wel bekend als mevrouw Bastiaans, wie weet zal het in de toekomst misschien toch nog… ???

Monique, mijn lieve P.A., wat ben je toch een heerlijk mens. Recht voor z'n raap en dat alles heerlijk op z'n Haags. Wat is het toch mooi als je kunt communiceren zonder ook maar één woord te zeggen. Wat hebben we een hoop afgelachen en wat zullen we dat nog vaak gaan doen. Want of we nou op het lab zijn of in Den Haag om met o.a. Edward een 'bakkie' te doen, het is altijd gezellig. Bedankt voor wie je bent en bedankt voor het feit dat je altijd voor me klaar staat.

Uiteraard wil ik alle andere collega's ook ontzettend bedanken voor al die mooie momenten in de afgelopen jaren. Mensen van de AIZ, bedankt dat jullie ons als klein researchgroepje wilden adopteren. Jullie input wordt zeer gewaardeerd. Diana, zowel de BBQ's als de carnavalsoptochten in Prinsenbeek waren iedere keer weer top! De vrijmibo's en lunches met de mensen van de AID waren ook altijd leuk. Marco, jij in het bijzonder nog bedankt voor het feit dat je af en toe back-up begeleider wilde zijn. Je weet het: 'we do make the difference!'. Ingrid Pico, zoals jij overal een feestje van weet te maken, dat kan er maar één. Vooral blijven doen! Dames bij de frontservice, we zagen elkaar niet heel vaak maar we hebben ook heel wat afgelachen samen. Keep up the good work! Dames van het secretariaat, jullie ook bedankt voor alle goede zorgen en behulpzaamheid. MJ's, ook jullie bedankt voor de gezelligheid en hulpvaardigheid. Aan

alle anderen waarvan ik de naam niet heb genoemd, weet dat ik heel erg genoten heb van mijn tijd bij de afdeling Immunologie en daar heb jij zeker aan bijgedragen.
Ook wil ik nog graag mijn vrienden bedanken voor alle steun en support de afgelopen jaren. Of we elkaar nou veel zagen of niet, op jullie kon ik rekenen! Bedankt voor die talloze mooie momenten. Jullie zijn allemaal kanjers. Het is niet mogelijk om iedereen hier te noemen maar een paar mensen wil ik toch graag in het bijzonder bedanken. Lieve Suus en Miek, in 2001 werden we huisgenoten en later inwoners van Den Haag. Inmiddels hebben we alles wel van elkaar gezien, gehoord en meegemaakt, en dat schept een hele sterke band. Suus, hoe onze carrières ook zullen gaan verlopen, ons 'navelstrengetje' zal ons altijd blijven binden. Miek, als rotsen in de branding hebben we veel voor onze kiezen gehad maar we staan nog steeds overeind. En hoe!! Jeroen, wij delen eenzelfde interesse in optimale vrijetijdsbesteding. Zo ervaren als jij zal ik nooit worden maar dat zal de pret absoluut niet drukken. Lieve Kaat, door dik en dun, je was er altijd wanneer ook nodig. Zelfs nu je in België woont, weten we elkaar nog genoeg te vinden. Dergelijke afstanden zijn maar relatief en je bent het meer dan waard! Karolina, Tsutsie, ooit begonnen we als collega's bij Add2X en nu kom ik niet meer van je af. Gelukkig maar, want je bent er één uit duizenden. Flip, pretletter, heerlijk zoals jij in het leven staat: All-in en gaan met die banaan! Stiekem benijd ik je daar wel eens om. Vanaf nu kan ik meer tijd investeren in het afkijken van deze kunst. Anne Marie, jouw enthousiasme en drive werken zo aanstekelijk. Recht op je doel af en het lukt je steeds weer. Tegenwoordig zien we elkaar niet zo vaak meer maar gelukkig hebben we nog steeds onze culinaire en culturele uitstapjes die dat meer dan goed maken. Bas, ooit begonnen we als bestuursgenoten bij SSR-W en over tig jaar eindigen we, daar waar we ooit begonnen zijn, op de vloer naast de bar, dan van je eigen brouwerij die je gegarandeerd nog eens gaat opzetten. Beter zouden we het haast niet kunnen treffen.
En tot slot mijn familie met in het bijzonder mijn lieve Muti. Al begrijp je helemaal niets van alles wat ik de afgelopen jaren heb gedaan, zonder jouw support had ik nooit kunnen bereiken wat ik nu heb bereikt. Zeker het afgelopen jaar, wat voor ons beiden erg zwaar was, stond je voor me klaar. Het was een lange rit, maar ik ben er! Bedankt!

9

Chapter 10
About the author

Curriculum Vitae
Bibliography
PhD portfolio

Curriculum Vitae

Jeroen Bastiaans was born in 's Hertogenbosch on June 7th 1980. In 1997 he graduated from secondary school (MAVO) at the d' Oultremontcollege in Drunen. Hereafter he attended the biochemistry program at the Middelbare Laboratorium Opleiding (MLO) at ROC de Leijgraaf in Oss. During this study he did an internship at department of Biotechnology at Numico Research B.V., Wageningen, The Netherlands (supervisors Dr. A. Mensink and D. Jeukens) in which he participated in the 'Synbiotica' project. After graduating in 2001 he continued his studies by attending the biotechnology program at the Hogere Laboratorium Opleiding (HLO) at the Hogeschool Arnhem en Nijmegen (HAN) in Nijmegen. He did an internship at the faculty of Agrotechnology & Food Innovations, Wageningen UR, University and Research center, Wageningen, The Netherlands (supervisors Dr. F.A. de Wolf and M.W.T. Werten) entitled '*the production of custom-designed biologically degradable plastics by Pichia pastoris*'. After obtaining his bachelor's degree in 2004, he got a full-time position in the board of Student Association SSR-W in Wageningen till 2005. From 2005 till 2008 he was employed as research technician at Add2X Biosciences B.V., a spin-off company within the faculty of Biology, Leiden University, Leiden, The Netherlands (supervisors Dr. R. Offringa and Dr. P. de Boer). There he was involved in the development of a highly efficient knock-in and knock-out gene targeting strategy in yeast and plants. From 2009 till 2011 he was employed by the Rotterdam Eye Hospital to work as research technician at the department of Immunology, Erasmus University Medical Center, Rotterdam, The Netherlands (supervisors Prof. dr. P.M. van Hagen and Dr. W.A. Dik) during which he studied the role of the neonatal Fc-receptor in retinal pigment epithelial cells. In 2011 he started with his PhD-project entitled '*the role of coagulation proteins in proliferative vitreoretinopathy*' also at the department of Immunology in collaboration with the Rotterdam Eye Hospital (supervisors Prof. dr. J.C. van Meurs, Prof. dr. P.M. van Hagen and Dr. W.A. Dik). During his research, Jeroen was active in several committees, supervised students, attended several courses and workshops, assisted in practical courses for medical students and presented the results of his research at national and international conferences in the fields of both Immunology and Ophthalmology. After finishing his PhD-project he will continue his research by studying the effects of fibrinopeptides on (auto-)inflammatory responses by RPE, for which a grant was obtained via the International Retinal Research Foundation.

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van den Berg JW, van der Zee M, de Bruin RW, van Holten-Neelen C, **Bastiaans J**, Nagtzaam NM, IJzermans JN, Benner R, Dik WA.
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Book chapter

Dik WA, van Laar JAM, Smits - te Nijenhuis M, Kolijn - Couwenberg MJ, Nagtzaam NMA, **Bastiaans J**, Kamphuis L, Kuijpers RWAM, van Hagen PM, Hooijkaas H.
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Nieuwe Ontwikkelingen in de Medische Immunologie 2010. ISBN: 978-90-73436-91-6

PhD portfolio

Name PhD student:	Jeroen Bastiaans
Erasmus MC Department:	Immunology
Research School:	Molecular Medicine (MolMed)
PhD period:	July 2011 – September 2015
Promotores:	Prof.dr. J.C. van Meurs, Prof.dr. P.M. van Hagen
Co-promotor:	Dr. W.A. Dik

PhD training

In depth courses and workshops

2012	Advanced molecular immunology (MolMed)
2012	SPSS statistics (MolMed)
2012	Research management for PhD students (MolMed)
2012	Adobe Photoshop & Illustrator CS5 (MolMed)
2013	Presenting skills (MolMed)
2013	Management (NIBI)
2013	Writing successful grant proposals (MolMed)
2014	Medical Immunology (Dept. of Immunology, Erasmus MC)
2014	Biomedical English writing (NIHES)

National conferences

2011	The Association for Research in Vision and Ophthalmology - Nederland (ARVO-Ned), Utrecht - *oral presentation*
2011	Nederlandse Vereniging voor Immunologie (NVvI), Noordwijkerhout - *poster presentation*
2012	Molecular Medicine Day, Rotterdam - *poster presentation*
2012	Dutch Ophthalmology PhD-Students (DOPS), Nijmegen - *oral presentation*
2012	Symposium Sarcoidosis & IPF, Rotterdam
2012	Nederlandse Vereniging voor Immunologie (NVvI), Noordwijkerhout - *poster presentation*
2013	Dutch Ophthalmology PhD-Students (DOPS), Nijmegen - *moderator*
2013	Molecular Medicine Day, Rotterdam - *poster presentation*
2013	Nederlandse Vereniging voor Immunologie (NVvI), Noordwijkerhout - *poster presentation*
2014	Science days - Internal Medicine Erasmus MC, Antwerpen - *poster presentation*
2014	Dutch Ophthalmology PhD-Students (DOPS), Nijmegen - *oral presentation*
2014	Nederlandse Vereniging voor Immunologie (NVvI), Kaatsheuvel - *poster presentation*
2015	Dutch Ophthalmology PhD-Students (DOPS), Nijmegen - *moderator*
2015	Medicine Day, Rotterdam - *elevator pitch + poster presentation*

International conferences

2011	The Association for Research in Vision and Ophthalmology (ARVO), Fort Lauderdale, Florida, USA - *poster presentation*
2012	The Association for Research in Vision and Ophthalmology (ARVO), Fort Lauderdale, Florida, USA - *poster presentation*
2012	The European workshops on Immune-Mediated Inflammatory Diseases (ewIMID), Noordwijk, The Netherlands - *poster presentation*
2013	The International Congress of Immunology (ICI), Milan, Italy - *oral presentation*
2014	The International Ocular Inflammation Society (IOIS), Valencia, Spain - *oral presentation*
2014	The Association for Research in Vision and Ophthalmology (ARVO), Orlando, Florida, USA - *poster presentation*

Other

2011	Internal auditor training (ISO9001:2008 by Lloyds)
2012	Refreshment course for internal auditors (ISO9001:2008 by Lloyds)
2013	Organizing committee of the 17th Molecular Medicine day
2014	Internal auditor training (ISO15189:2012 by QAducation)
2014	Organizing committee of the 18th Molecular Medicine day
2011 - present	PhD committee of the postgraduate school of Molecular Medicine

At the department of Immunology

2011 - 2012	Technical staff
2012 - 2013	Activity/party committee
2013 - 2014	PhD committee
2011 - present	Internal auditor (quality control)
2011 - 2014	Attending journal clubs
2011 - present	Attending department and research meetings
2011 - present	Attending seminars and mini-symposia

Memberships

2011 - present	Nederlandse Vereniging voor Immunologie (NVvI)
2011 - present	The Association for Research in Vision and Ophthalmology (ARVO)

Grants

2011	NVvI Travel grant to attend the ARVO conference in Fort Lauderdale
2014	International Retinal Research Foundation (co-author)

Teaching

2013 - present	Practical course Immunology (vaardigheidsonderwijs) 2nd year medical students
2011 - 2014	Supervising interns for BSc and MSc thesis

Courses and workshops (Desiderius school, Erasmus MC)

2014	Teach the teacher training
2014	Supervision of individuals
2014	Providing feedback
2014	Managing big and small groups