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List of Contributors

Paul N. Bishop
University of Manchester
Manchester Royal Eye Hospital
Oxford Road
Manchester M13 9WH (UK)

David G. Charteris
Moorfields Eye Hospital
City Road
London EC1V 2PD (UK)

Carlos E. Cury, Jr.
Vision Institute, Department of Ophthalmology
Federal University of Sao Paulo
Rua Presidente Coutinho 579
conj 501
Florianópolis
SC 88015–300 (Brazil)

Kimberly A. Drenser
Eye Research Institute, University Oakland
3535 West 13 Mile Road, Suite 344
Royal Oak, MI 48073 (USA)

Kirsten H. Eibl
University Eye Hospital LMU
Ludwig-Maximilians-University
Mathildienstr. 8
DE-80336 Munich (Germany)

Michel E. Farah
Vision Institute, Department of Ophthalmology
Federal University of Sao Paulo
Rua Presidente Coutinho 579
conj 501
Florianópolis
SC 88015–300 (Brazil)

Steven K. Fisher
Neuroscience Research Institute
University of California
Santa Barbara, CA 93106-5060 (USA)

Patrice E. Fort
Penn State Hershey Eye Center and
Department of Cellular and Molecular
Physiology
Penn State College of Medicine
500 University Drive, HU097
Hershey, PA 17033 (USA)

Arnd Gandorfer
Vitreoretinal and Pathology Unit
Augenklinik der Ludwig-Maximilians-
Universität
Mathildenstrasse 8
DE–80336 Munich (Germany)

Thomas W. Gardner
Penn State Hershey Eye Center and
Department of Cellular and Molecular
Physiology
Penn State College of Medicine
500 University Drive, HU097
Hershey, PA 17033 (USA)

David T. Goldenberg
3535 W. 13 Mile Rd., Suite 344
Royal Oak, MI 48073 (USA)
Hisanori Imai  
Penn State Hershey Eye Center and  
Department of Cellular and Molecular  
Physiology  
Penn State College of Medicine  
500 University Drive, HU097  
Hershey, PA 17033 (USA)  

Motohiro Kamei  
Department of Ophthalmology  
Osaka University Medical School  
2–2 Yamadaoka  
Suita, 565-0871 (Japan)  

Baruch Kuppermann  
UC Irvine Medical Center  
Ophthalmology Clinic  
101 The City Drive South  
Orange, CA 92697 (USA)  

Geoffrey P. Lewis  
Neuroscience Research Institute  
University of California  
Santa Barbara, CA 93106-5060 (USA)  

Carsten H. Meyer  
Department of Ophthalmology  
University of Bonn  
Ernst-Abbe-Strasse 2  
DE–53127 Bonn (Germany)  

Raja Narayanan  
Smt Kanuri santhamma Vitreoretina center  
L.V. Prasad Eye Institute  
L.V. Prasad Marg, Banjara Hills  
Hyderabad 500034 (India)  

Eduardo B. Rodrigues  
Vision Institute, Department of Ophthalmology  
Federal University of Sao Paulo  
Rua Presidente Coutinho 579  
conj 501  
Florianópolis  
SC 88015–300 (Brazil)  

Ravi S.J. Singh  
Penn State Hershey Eye Center and  
Department of Cellular and Molecular  
Physiology  
Penn State College of Medicine  
500 University Drive, HU097  
Hershey, PA 17033 (USA)  

Yasuo Tano  
Department of Ophthalmology  
Osaka University Medical School  
2–2 Yamadaoka  
Suita, 565-0871 (Japan)  

Michael T. Trese  
3535 W. 13 Mile Rd., Suite 344  
Royal Oak, MI 48073 (USA)  

Louisa Wickham  
Moorfields Eye Hospital  
City Road  
London EC1V 2PD (UK)
In memoriam of Professor Yasuo Tano, who suddenly passed away on 31 January 2009.
More than 35 years have passed since the advent of pars plana vitrectomy, and vitreo-retinal surgery has developed to highly sophisticated techniques to treat retinal diseases. Removal of the vitreous gel and hemorrhage not only clears the optical axis of the eye, but enables the surgeon to approach the retina and the vitreoretinal interface directly, thereby relieving traction and removing pathological tissue, such as epiretinal membranes. Peeling off the internal limiting membrane has proven to be a safe and effective technique in macular surgery, resulting in macular hole closure. Twenty years ago, nobody would have imagined this. Today, final success rates beyond 90% can be achieved in macular and reattachment surgery.

However, there are limitations of current vitreoretinal surgery techniques, which are mechanically based. Removal of the vitreous is incomplete, especially at the vitreo-retinal interface and at the vitreous base. This may lead to persistent or recurrent traction on the retina, resulting in retinal tear formation or reproliferation. More aggressive removal of the vitreous by mechanical means, however, carries the risk of damaging the retina. When epiretinal membranes are removed in PVR cases and in diabetic eyes with traction retinal detachment, gliotic scar tissue is removed but neural retina is not treated. Thus, despite anatomical reattachment, visual results are often disappointing.

Pharmacology-assisted vitreoretinal surgery can help to overcome these limitations. There are enzymes which cleave the vitreoretinal junction without damaging the retina, and those for liquefaction. Thereby, vitreolysis and induction of posterior vitreous detachment has become possible without the need for vitrectomy. Recent results from clinical trials show that up to 40% of eyes achieve release of traction without surgery.

Pharmacologic vitreolysis will change our current indications and concepts in treating retinal and macular diseases, and earlier intervention might save visual function before advanced stages have destroyed the retinal cytoarchitecture. In diabetic eyes, for example, enzymatic PVD induction at an early stage of the disease might inhibit fibrovascular and fibrocellular proliferations at the vitreoretinal interface, thereby preventing progression to proliferative disease.
Neuroprotective and antiproliferative agents may hold the promise of preserving neuronal function when the retina is detached or when PVR has developed. This again may change the time point of intervention from currently advanced stages to an earlier disease stage with less pathology. Fibrinolytic and antiproliferative agents help to treat disasters in ophthalmology such as massive submacular hemorrhage or retinal detachment in retinopathy of prematurity.

It is now time to combine pharmacological concepts and vitreoretinal surgery. World renowned experts and opinion leaders in their field have contributed their knowledge and skills to make this book the first summary on pharmacology-assisted vitreoretinal surgery. I am greatly indebted to the authors, and my hopes are that this book was a basis and motivation for clinicians and researchers who want to bring retinal surgery further by introducing pharmacology-assisted vitreoretinal surgery.

Arnd Gandorfer
Munich, 2009
Objective of Pharmacologic Vitreolysis

Arnd Gandorfer

Vitreoretinal and Pathology Unit, University Eye Hospital Munich, Ludwig-Maximilians University, Munich, Germany

Abstract

The goal of pharmacologic vitreolysis is to cleave the vitreoretinal junction, thereby inducing posterior vitreous detachment (PVD), and to liquefy the vitreous gel. There are several reasons to pursue a pharmacologic approach: (1) Mechanical vitrectomy is incomplete. Both at the posterior pole and in the retinal periphery, remnants of the cortical vitreous are left behind at the internal limiting membrane of the retina, causing vitreoretinal traction and (re)proliferation of cells, and leading to surgical failure. (2) Pharmacologic vitreolysis offers complete PVD without mechanical manipulation at the vitreoretinal interface, such as ILM peeling, thereby minimizing the risk of iatrogenic damage to the macula. (3) An intravitreal injection resulting in complete PVD is a less traumatic approach than vitrectomy, and it might be beneficial as prophylactic treatment regime in retinal diseases characterized by fibrocellular and fibrovascular proliferation at the vitreoretinal interface, such as diabetic macular edema and proliferative retinopathy, in order to prevent advanced stages of disease. (4) Cleaving the cortical hyaloid completely from the retina changes the molecular flux across the vitreoretinal interface and improves oxygen supply to the retina, a major mechanism of action which might significantly interfere with biochemical pathways of retinal hypoxia, leading to an overexpression of vasoactive substances such as vascular endothelial growth factor.

The vitreous, and in particular the vitreoretinal interface, plays an important role in the pathogenesis of many retinal disorders. An abnormal interface has been implicated in vitreomacular traction syndrome, macular holes, diabetic macular edema, proliferative diabetic retinopathy, retinal detachment, and proliferative vitreoretinopathy [1]. A common finding in these entities is a vitreous which is firmly attached to the retina, thereby preventing complete posterior vitreous detachment (PVD) [2, 3]. Even in cases of vitreous liquefaction, the vitreous cortex remains attached to the retina, forming so-called ‘vitreoschisis’, which acts as a scaffold for fibrocellular and fibrovascular proliferations [4–6]. This may increase traction on the retina leading to significant patient morbidity and surgical failure.
Vitrectomy is the treatment modality of choice in these patients, and complete removal of the cortical hyaloid is critical for success of the operation [2, 7]. However, mechanical separation of the vitreous from the retina is incomplete, as cortical vitreous fibrils are left behind on the internal limiting membrane (ILM) of the retina (fig. 1) [6]. Removal of the ILM creates a new vitreomacular interface by eliminating the vitreous cortex and proliferative tissue (fig. 2). However, ILM removal requires direct
manipulation of the macula which can potentially result in retinal damage, leading to functional defects, such as central or paracentral scotomata [8, 9]. Ultrastructural analysis of peeled ILM specimens has shown damage to Müller cells and avulsion of macular ganglion cells (fig. 3) [8, 10]. Although today, ILM peeling appears safe in clinical terms, it may not be the best option ever in terms of safety and visual benefit.

Cleaving the vitreoretinal interface at the vitreal side of the ILM would be a logical approach to relieve traction, as the ILM itself cannot exert traction (fig. 4). Traction is generated by contractile cells at the vitreoretinal interface [11]. In most instances, these cells proliferate on a layer of vitreous cortex which remained attached at the ILM after incomplete PVD [5, 12, 13]. From an ultrastructural point of view, separation of
the cortical vitreous completely from the ILM would eliminate the cellular proliferations, thereby relieving the traction they generate.

Complete separation of the vitreous from the retina by enzymatic means would remove the pathologic tissue from the retina, and eliminate the scaffold for cellular re-proliferation [14–17]. Especially diabetic eyes are characterized by a firm vitreoretinal attachment and a thickened and taut vitreous cortex which frequently contains cellular proliferations (fig. 5) [3, 12]. Given that some cells have been shown to produce vasoactive and vasoproliferative substances, such as vascular endothelial growth factor and others, complete removal of the vitreous cortex with embedded cells could lead back to a more physiological state of the vitreomacular interface in patients with diabetic macular edema [18].

Moreover, a clean ILM without remnants of the cortical vitreous would hypothetically prevent the growth of fibrovascular membranes out of the retina, as these newly formed vessels commonly proliferate within and along the cortical hyaloid [19]. Given that a complete PVD was achieved by a relatively atraumatic intravitreal injection and not by an operation such as vitrectomy, pharmacologic vitreolysis could be discussed in select cases as a prophylactic treatment regime to prevent proliferative diabetic retinopathy [2].

Another mechanism of action of enzymatically induced PVD and vitreous liquefaction is an alteration of molecular flux into the vitreous cavity, out of the vitreous cavity, and across the vitreoretinal interface [20]. It has been know since the 1980s that
oxygen levels within the vitreous cavity are higher in vitrectomized eyes compared to nonvitrectomized eyes [21]. Recently, it has been demonstrated that the oxygen supply of the retina can be modified by a microplasmin-induced PVD and vitreous liquefaction [20]. It is of note that liquefaction alone did not have this effect. Obviously, the vitreous cortex acts as a barrier for molecules when crossing the vitreoretinal border, and this may not only be the case for oxygen but also when other molecules are produced within the eye or even when are injected intravitreally [22]. A better oxygen supply of the retina addresses both diseases with hypoxia such as diabetic retinopathy and retinal vein occlusion, both complicated by macular edema and neovascularization, and those in which anti-VEGF treatment is performed in order to decrease intravitreal VEGF levels such as age-related macular degeneration. Interestingly, eyes with wet AMD also show a significantly higher percentage of attached vitreous at the macula compared to non-/dry AMD eyes. Separation of the posterior hyaloid from the retina and liquefaction alters the molecular flux across the vitreoretinal interface, and it might be expected that larger molecules are even more affected by the barrier function of the cortical vitreous than small molecules such as oxygen.

The summary of potential objectives of pharmacologic vitreolysis includes complete vitreoretinal separation resulting in a clean ILM, less manipulation at the vitreoretinal interface compared to current vitrectomy techniques, a well-defined cleavage plane at the vitreal side of the ILM to remove fibrocellular traction, better oxygen supply due to an alteration of the molecular flux, and a less traumatic and maybe prophylactic treatment regime in select cases in the future [2, 23, 24].

References


Vitreous as a Substrate for Vitreolysis

Paul N. Bishop

University of Manchester and Manchester Biomedical Research Centre, Manchester Royal Eye Hospital, Manchester, UK

Abstract

There is an increasing interest in the concept of using enzymes to induce vitreoretinal separation and/or modulate the structure of the vitreous as an aid to, or replacement for, mechanical vitrectomy. In order to understand how these enzymes work and to develop new and better therapeutics, knowledge of vitreous structure at a molecular and supramolecular level is key. The vitreous like many extracellular matrices has a composite structure with a network of collagen fibrils, that is essential for the gel structure, and a glycosaminoglycan network composed predominantly of hyaluronan that fills the spaces between the collagen fibrils and helps to inflate the matrix. The collagen fibrils are very resistant to proteolytic degradation so these do not make a good target for pharmacological vitreolysis, but degradation of the hyaluronan facilitates the dispersal of vitreous haemorrhage. The molecular mechanisms underlying postbasal vitreoretinal adhesion are poorly understood but it is likely that intermediary molecules that link cortical vitreous collagen fibrils to the inner limiting lamina play a major role. Furthermore, it is apparent that proteolytic enzymes such as plasmin fragment these linking molecules and thereby facilitate vitreoretinal disinsertion.

There has been considerable interest in recent years in the use of pharmacological agents to facilitate, or indeed replace mechanical vitrectomy. The main research focus has been on enzymes that cleave the vitreoretinal interface, modify vitreous structure, or a combination of the two. These agents have the potential to facilitate the treatment of conditions where there is vitreoretinal traction or to create a posterior vitreous detachment (PVD) to protect against pathologies such as proliferative diabetic retinopathy. Alternatively, agents that modify gel structure may be used, for example, to facilitate the clearance of blood from the vitreous cavity.

The use of enzymes in the ocular cavity has a long history with, for example, chymotrypsin being used to disrupt the zonules during intracapsular cataract surgery. Interestingly this did not appear to cause significant collateral damage to ocular tissues. In the 1970s, experiments were undertaken to determine whether bacterial collagenases could be used for vitreolysis, but these were abandoned when it became
apparent that intravitreal collagenase caused collateral damage to the retinal vasculature. These early experiments did however focus attention on one of the key issues in this research field, i.e. to identify pharmacological agents that assist or replace mechanical vitrectomy, but do not cause collateral damage to other ocular structures. To achieve this aim it is helpful to understand, at a molecular level, how the vitreous and vitreoretinal interface are organised, so that specific molecules or molecular interactions can be targeted with appropriate pharmacological agents.

Structural Molecules and Macromolecular Organisation of the Vitreous Humour

Structural Macromolecules

The vitreous humour is a unique extracellular matrix that normally only contains only a few cells, mainly hyalocytes, that are localised in its periphery. Extracellular matrices are formed and maintained by network-forming macromolecules with complementary properties. Proteins such as collagens endow tissues with shape, strength, flexibility and resistance to tractional forces. Carbohydrates, particularly glycosaminoglycans (GAGs), fill the spaces between the collagens, inflate the tissue and resist compressive forces. GAGs are formed from long chains of negatively charged sugar units that attract counter-ions and water and thereby provide a swelling pressure that spaces apart and stabilises the collagen network. In the vitreous the major components of this composite structure are fibrillar collagens and the GAG hyaluronan (fig. 1).

Collagens and Associated Molecules

The vitreous gel contains a low concentration of collagen that is estimated to be 300 μg/ml in human eyes [1]. Despite this low concentration, it is the collagen fibrils that impart gel-like properties to the vitreous. In 1982, Balazs and Denlinger [2] showed that the total amount of vitreous collagen does not change throughout life so the concentration decreases as the eye grows; they therefore suggested that there is no postnatal synthesis of vitreous collagen. However, more recent research suggests that there is some postnatal synthesis of collagen, although probably at a low-level [1, 3–5].

Collagen molecules are formed by three polypeptide chains (α-chains) that fold in a characteristic triple-helical configuration [1]. This triple-helical structure requires that every third amino acid is a glycine residue, so collagen α-chains have (Gly-X-Y)_n triplet amino acid repeats where X and Y can be any amino acids, but are frequently proline and hydroxyproline. Hydroxyproline is important in stabilising the structure of the collagen triple helix by forming extra hydrogen bonds. The collagen molecules assemble into fibrils and the amino acids lysine and hydroxylsine then participate in crosslink formation within the fibrils. The net result is the formation of very strong
rope-like structures that are resistant to the actions of many proteolytic enzymes. Enzymes that are known to breakdown collagen include bacterial collagenase, some metalloproteinases and cathespin K.

There are at least 27 different types of collagen molecules (i.e. collagen types I–XXVII) and these typically assemble into fibrils or sheet-like structures. In the vitreous humour nearly all of the collagen is in the form of thin, uniform, heterotypic (of mixed composition) fibrils containing collagen types II, IX and V/XI [1, 6]. It is of note that cartilage collagen fibrils have a very similar composition. There are also type VI collagen microfibrils in the vitreous, but they are in low abundance and their significance is unknown [1].

The predominant component of the heterotypic collagen fibrils (60–75%) is type II collagen [1]. Molecules of this collagen are composed of three identical α-chains, i.e. its composition is α1(II)3. When fibrillar collagens such as type II collagen molecules are secreted into the extracellular environment they are in a soluble precursor form, the procollagen. Procollagen has terminal extensions called the N- and C-propeptides. Once within the extracellular environment, the procollagen is 'processed' by specific enzymes called N-proteinase and C-proteinase (or BMP-1) that cleave the N- and C-propeptides respectively, leaving small non-collagenous telopeptides at each end of the triple-helical region. This process reduces the solubility of the collagen molecules and allows them to participate in fibril formation.

Type IX collagen has been estimated to represent up to 25% of the collagen in the heterotypic collagen fibrils of vitreous [1]. It covalently links to the surface of these
fibrils in a regular periodicity (the D-period). It is a member of the family of fibril associated collagens with interrupted triple helices (FACIT), and a common feature of these collagens is a multiple-domain structure with triple-helical domains (COL domains) that are separated or interrupted by short non-triple-helical domains (NC domains). Type IX collagen is a heterotrimer made of 3 distinct polypeptide chains i.e. its chain composition is α1(IX)α2(IX)α3(IX) and these assemble to produce three collagenous domains (COL1, COL2 and COL3) interspersed between four non-collagenous domains (NC1, NC2, NC3 and NC4). In vitreous, the type IX collagen is a proteoglycan and is synthesised with a single covalently-linked chondroitin sulphate GAG chain. These chondroitin sulphate chains can be visualised by electron microscopy after labelling with the cationic dye cupromeronic blue (fig. 2).

The third collagen in the heterotypic collagen fibrils is another fibrillar collagen called type V/XI collagen and this represents 10–25% of the collagen in the fibrils [1]. It co-assembles with the type II collagen to form the core of the fibrils. This collagen contains α1(XI) and α2(V) chains, probably with a stoichiometry of α1(XI)2α2(V). The N-terminal domain of type XI collagen is incompletely removed by processing and may play a role in regulating the fibril diameter.

The heterotypic collagen fibrils of the vitreous are coated with a molecule called opticin [7, 8]. This is a member of the extracellular matrix small leucine-rich repeat protein/proteoglycan (SLRP) family. Most members of this family are proteoglycans, but opticin is instead glycosylated via a cluster of O-linked oligosaccharides. Opticin exists as a dimer in solution as a result of interactions between the LRR domains [9]. Opticin binds to GAGs including heparan sulphate and chondroitin sulphate and

![Fig. 2. Electron micrographs of vitreous collagen fibrils stained with uranyl acetate (labelling collagen fibrils) and cupromeronic blue (labelling the chondroitin sulphate chains of the type IX collagen). The collagen fibrils are organised into bundles and within these bundles the type IX collagen chondroitin sulphate chains appear to bridge between adjacent fibrils in a ladder-like configuration. Therefore, these chondroitin sulphate chains may both space apart and interconnect the collagen fibrils.](image-url)
may thereby play a role in stabilising the vitreous gel structure and in adhesion at the vitreoretinal interface [10].

**GAGs and Proteoglycans**

GAGs are composed of long chains of repeating disaccharide units. All GAGs except hyaluronan are synthesised attached to a protein core (thereby forming proteoglycans) and undergo varying degrees of modification during their synthesis including sulphation, epimerisation and acetylation/deacetylation. The predominant GAG in vitreous is hyaluronan (>90%), but it also contains small amounts of chondroitin sulphate that may be important to its structure.

Hyaluronan is formed from repeating disaccharide units \([\beta1–4\ \text{glucuronic acid } \beta1–3\ N\text{-acetylglucosamine}]_n\). Chains of hyaluronan can be very long and form networks through entanglement. It is distinguished from the other GAGs in that it is not synthesised covalently linked to a core protein and it is not sulphated. Hyaluronan is not uniformly distributed within the vitreous and the highest concentration is found in the posterior vitreous cortex. In the adult human vitreous the hyaluronan concentration has been estimated to be between 65 and 400 μg/ml and the average molecular weight to be 2–4 million [1].

The repeating disaccharide unit of chondroitin sulphate chains is \([\beta1–4\ \text{glucuronic acid } \beta1–3\ N\text{-acetylgalactosamine}]_n\). Sulphation is added to these chains at various points. The vitreous has been shown to contain two chondroitin sulphate proteoglycans, type IX collagen (see above) and versican [11]. Versican is a large proteoglycan with a central domain that carries multiple chondroitin sulphate chains. It possesses an N-terminal hyaluronan-binding domain and the binding to hyaluronan is stabilised by link protein, which has also been identified in the vitreous. Therefore, it is likely that the versican in vitreous is linked to the hyaluronan network.

GAGs can be degraded by specific enzymes. Hyaluronidases, also called hyaluronan lyases, are enzymes that degrade hyaluronan. They are derived from different sources and have different specificities. For example, testicular hyaluronidase (bovine or ovine) degrades chondroitin and dermatan sulphate as well as hyaluronan, and some preparations have significant protease activity. By contrast, the hyaluronidase derived from *Streptomyces* is specific for hyaluronan. The enzyme chondroitin ABC lyase degrades chondroitin and dermatan sulphate, and it addition has some activity against hyaluronan.

**Supramolecular Organisation**

The gel state of the vitreous is maintained by the network of heterotypic collagen fibrils as their removal, for example by centrifugation, converts the vitreous into a viscous liquid. This network of collagen fibrils can be observed by electron microscopy.
Transmission electron microscopy reveals that these collagen fibrils are unusually thin, approximately 15 nm in diameter, and unbranched [6]. Analyses by freeze-etch rotary shadowing electron microscopy showed that the collagen fibrils are arranged in bundles within the vitreous and form an extended interconnected network by branching between these bundles [12]. In the young vitreous, the collagen fibrils within these bundles appear to run closely together in parallel, but are not fused (figs. 2, 5). Electron microscopic studies, using cationic dyes, suggest that the chondroitin sulphate chains of type IX collagen play a role in both connecting together and spacing apart the collagen fibrils within these bundles [13].

Hyaluronan provides a swelling pressure to the collagen network and hence the vitreous gel. However, the enzymatic degradation of the hyaluronan in isolated bovine vitreous gels resulted in some gel shrinkage, but not complete collapse; so hyaluronan is not a prerequisite for the bovine vitreous gel structure [14]. Further studies demonstrated that hyaluronan weakly associates with vitreous collagen fibrils and the hyaluronan network ‘stiffens’ the collagenous network, as hyaluronidase digestion resulted in deflation and relaxation of the collagen network [12]. Taken together these data suggest that the gel structure can exist without hyaluronan, but that it is deflated. Therefore, hyaluronidases are unlikely to cause extensive liquefaction of the vitreous. However, hyaluronan contributes towards the trapping of cells in the vitreous cavity, for example following vitreous haemorrhage, and the enzymatic depolymerisation of hyaluronan facilitates the dispersion of trapped cells.

**Vitreoretinal Adhesion**

Morphological analyses reveal that the mechanisms underlying vitreoretinal adhesion differ within and posterior to the vitreous base. Within the vitreous base there is effectively an unbreakable adhesion and this is a result of the vitreous collagen fibrils passing through defects in the inner limiting lamina (ILL) and intertwining with collagen on the cellular side of this basement membrane [3]. Indeed the interlinking of newly synthesised collagen in the peripheral retina that has penetrated the ILL with the pre-existing cortical vitreous collagen may account for the posterior migration of the posterior border of the vitreous base with aging [3].

Posterior to the vitreous base the cortical vitreous collagen fibrils do not generally insert into or pass through the post-oral ILL [1]. However, there may be focal areas where direct collagenous connections are formed through the ILL such as in strong paravascular vitreoretinal adhesions [15]. Nonetheless, for most of the post-oral vitreoretinal interface, the cortical vitreous collagen fibrils run parallel with the ILL and the basis of adhesion is likely to be interactions between molecules on the surface of the cortical collagen fibrils and inner surface of the ILL (fig. 3). The molecular basis of these adhesions is poorly understood. The ILL is a basement membrane that contains molecules including type IV collagen, laminins and
Vitreous and Vitreolysis

Heparan sulphate proteoglycans such as type XVIII collagen. It has been shown that collagen type XVIII contributes towards vitreoretinal adhesion in the mouse eye, as a proportion of eyes from type XVIII collagen knockout mice had vitreoretinal disinsertion [16]; this may have been due to abnormal development at the vitreoretinal interface or due to type XVIII collagen being directly involved in vitreoretinal adhesion. Type XVIII collagen is a heparan sulphate proteoglycan and opticin binds to heparan sulphate [10]. Therefore, it is possible that opticin on the surface of cortical vitreous collagen fibrils binds to heparan sulphate of the ILL including type XVIII collagen, thus providing a molecular basis for vitreoretinal adhesion. However, opticin knockout mice do not appear to have spontaneous vitreoretinal disinsertion [unpubl. obs.], suggesting that in the mouse eye this interaction is not critical for vitreoretinal adhesion and that other molecular interactions are also involved.

Vitreous Liquefaction and Posterior Vitreous Detachment

The human vitreous humour undergoes an inevitable process of liquefaction (or syneresis) with aging so that by the age of 80–90 years more than half of the vitreous is liquefied [1]. Ultrastructural studies have shown that collagen fibrils aggregate with aging into macroscopic strands within the vitreous gel [17]. Age-related vitreous liquefaction may be caused by this gradual and progressive aggregation of the collagen fibrils resulting in a redistribution of the collagen fibrils leaving areas devoid of collagen fibrils and thereby converted into a liquid, and the collagen aggregates concentrated in the residual gel (fig. 4, 5). An alternative hypothesis is that age-related liquefaction is a result of destruction of vitreous collagen fibrils through enzymatic activity [18].
There is an age-related loss of type IX collagen from the surface of the heterotypic collagen fibrils of the human eye [13]. Indeed, the half-life for the type IX collagen was found to be just 11 years of age. The chondroitin sulphate chains of type IX collagen appear to space collagen fibrils apart within the bundles that have been observed in the vitreous, so the age-related depletion of type IX collagen from the fibril surfaces will result in a loss of the spacers between the collagen fibrils. Once they come into contact, the stickiness of the fibrillar collagens will result in a tendency towards fusion. These conclusions are supported by a study showing that digestion of the vitreous with chondroitin ABC lyase, an enzyme that degrades the CS chains of type IX collagen, resulted in the aggregation of collagen fibrils [19]. Therefore, the aggregation of collagen that may result in vitreous liquefaction is caused by a loss of type IX collagen.

PVD is the process whereby the cortical vitreous gel splits away from the inner limiting lamina on the inner surface of the retina as far anteriorly as the posterior border of the vitreous base. It occurs spontaneously in approximately 25% of the population during their lifetime. PVD can be localised, partial or ‘complete’ (i.e. up to the posterior border of the vitreous base). PVD results from a combination of vitreous liquefaction and weakening of post-basal vitreoretinal adhesion. During PVD separation of the basal vitreous from the peripheral retina and ciliary body does not occur, because of the very strong adhesion in this region.

Whilst in a majority of subjects PVD occurs without major complications, in some it has sight-threatening complications. The concept of anomalous PVD was introduced by Sebag [20] to describe the situation where the extent of liquefaction exceeds weakening of vitreoretinal adhesion. This results in excessive tractional forces being exerted upon the retina during PVD and/or incomplete separation thereby leading

**Fig. 4.** During aging the vitreous collagen fibrils progressively aggregate together. This may lead to a redistribution of the collagen, with the aggregates collagen fibrils being concentrated in the residual gel structure and other areas becoming devoid of collagen resulting in their liquefaction.
to complications including haemorrhage, retinal tears and detachment, macular hole formation and vitreomacular traction syndrome.

**Enzymatic Vitreolysis**

There has been increasing interest over the past few years in the use of intravitreal enzymes to cleave the vitreoretinal interface and/or altering the structure of the vitreous. When intravitreal enzymes are used the dosing and duration of action in the vitreous cavity are important parameters to consider as too little enzyme will be ineffective and too much may cause collateral damage to other ocular tissues, particularly the retina. Different animal models have been used in recent studies, along with enucleated human eyes. Some of these studies have progressed to clinical trials.

*Plasmin, Microplasmin and Tissue Plasminogen Activator*

At the present time the most promising enzymes for inducing vitreoretinal separation are plasmin and microplasmin (Thrombogenetics Ltd., Dublin). Microplasmin is a recombinant protein that contains the catalytic domain of human plasmin. Plasmin and microplasmin are non-specific serine proteases that cleave a variety of glycoproteins including fibronectin, laminin, fibrin and thrombospondin. Whilst laminin and

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**Fig. 5.** The collagen fibrillar network of the vitreous and ageing changes. **a** The collagen fibrils (thick grey lines) form an extended network by being organised into small bundles that are interconnected by collagen fibrils running from one bundle to another. Within each bundle the collagen fibrils are both connected together and spaced apart by the CS chains of type IX collagen (thin black lines). **b** With ageing there is a loss of type IX collagen from the fibril surfaces. The loss of the type IX collagen CS chains from the fibril surfaces combined with an increased surface exposure of sticky type II collagen results in collagen fibrillar aggregation.
Fibronectin have been identified at the vitreoretinal interface and it has been shown that they are cleaved during plasmin-assisted vitrectomy [21], there is not direct evidence that these glycoproteins contribute towards vitreoretinal adhesion. It has been proposed that plasmin acts indirectly by cleaving endogenous (inactive) pro-metalloproteinases (pro-MMPs) and thereby converting them into the active MMP: Takano et al. [22] showed that intravitreal plasmin injection resulted in the activation of endogenous pro-MMP2. Indeed it has been shown that vitreous contains sufficient endogenous pro-MMP2 to cause significant destruction of the gel when activated [23].

In an investigation using post-mortem human eyes, plasmin was shown to induce complete vitreoretinal disinsertion detaching the cortical vitreous collagen from the inner limiting membrane as far as the posterior border of the vitreous base [24]. Scanning electron microscopy of the inner limiting membrane showed only sparse vitreous collagen fibrils remaining. Microplasmin increases vitreous diffusion coefficients, implying that it alters vitreous structure as well as inducing vitreoretinal disinsertion [25].

It was first reported by Verstraeten et al. [26] that when 1 U of plasmin was injected in rabbit eyes vitreous detachment was facilitated during vitrectomy. This group observed a transient decrease in electroretinogram b-wave amplitude, but no other adverse effects. Several studies supported these initial findings and subsequently clinical studies were performed autologous plasmin. Plasmin-assisted vitrectomy was performed in cases series with traumatic macular holes, diabetic retinopathy and adult stage 3 macular holes and in all of these studies autologous plasmin was reported to facilitate the vitrectomy [27–29]. Further studies supported the observations that autologous plasmin when delivered before a mechanical vitrectomy was safe and facilitated the procedure [30, 31].

Gandorfer et al. [32] evaluated the effects of 62.5–188 μg intravitreal microplasmin on post-mortem human eyes and 14.5–25 μg on feline eyes in vivo. 125–188 μg of microplasmin induced PVD in human eyes and 25 μg in the feline eyes and an ILL that was free of cortical vitreous collagen fibrils by scanning electron microscopy. There was no evidence of cellular damage to the feline retinas. Microplasmin was delivered by intravitreal injection into rabbit eyes in a dose range of 12.5–250 μg [33]. A transient inflammatory reaction and reduction in ERG was observed, with the effects on ERG being less at doses of less than 250 μg. Doses of 125 μg or higher induced PVD and an ILL devoid of collagen fibrils when examined by scanning electron microscopy. Thrombogenetics have instituted clinical trials to evaluate the safety and efficacy of microplasmin used in combination with mechanical vitrectomy (for vitreomacular traction), or used alone (for diabetic macular oedema). Trial reports suggest safety and efficacy, but these have yet to be published.

A further approach that has been used is to inject tissue plasminogen activator into the vitreous cavity. This converts endogenous vitreous plasminogen into plasmin. When 25 μg was injected shortly prior to mechanical vitrectomy it was reported to facilitate the procedure [34]. However, a subsequent study did not support these observations [35].
Dispase

Dispase is a neutral protease derived from *Bacillus polymyxa* that preferentially cleaves fibronectin and type IV collagen [36]. Initial studies using procine and human cadaver eyes [37] and then in vivo experiments using porcine eyes [38] suggested that intra-vitreal dispase could induce or facilitate PVD without causing significant collateral damage to other ocular tissues. However, subsequent studies found evidence of retinal haemorrhages, decreased electroretinogram responses, ultrastructural damage to the retina, cataract and epiretinal membrane formation following the intravitreal administration of dispase [39–41]. Therefore, these more recent studies bring into question the safety of this approach.

Hyaluronidase

Gottlieb et al. [42] investigated the effects of intravitreal hyaluronidase on the rabbit eye and concluded that it induced partial vitreolysis. A subsequent study by Harooni et al. [43] showed that hyaluronidase induced PVD, but this finding was not substantiated by other investigators [44]. A possible explanation for these differences is that preparations of hyaluronidase are often contaminated with significant amounts of proteolytic enzymes and that it was non-specific proteolysis that induced PVD in the Harooni study. A highly purified ovine hyaluronidase Vitrase™ (ISTA Pharmaceuticals, Calif., USA) has been developed for clinical use. Two phase III randomised controlled trials have shown that a single intravitreal injection of Vitrase is safe and demonstrated efficacy in aiding the clearance of vitreous haemorrhage [45, 46]. These studies did not report whether Vitrase induced PVD, but there was no propensity for retinal detachment in the Vitrase-treated group. A variable response was observed to Vitrase so a predictive model has been developed to identify those subjects that are most likely to have vitreous haemorrhage clearance by three months by analysing the patients at 1 month following treatment [47].

Chondroitinase

Intravitreal injection of chondroitinase (chondroitin ABC lyase) was reported to induce vitreoretinal separation in primates without any observed toxicity [48]. Following to this, a phase I clinical trial was initiated but the results were never published. Subsequently, the effects of chondroitin ABC lyase were investigated in porcine eyes and were reported to facilitate vitrectomy [49], but in a further study this enzyme was not observed to facilitate vitreoretinal separation during vitrectomy [50].
References


Paul N. Bishop
Manchester Royal Eye Hospital
Oxford Road
Manchester M13 9WH (UK)
Tel. +44 161 275 5755, Fax +44 161 275 3938, E-Mail paul.bishop@manchester.ac.uk
Hyaluronidase for Pharmacologic Vitreolysis

Raja Narayanan\textsuperscript{a} · Baruch D. Kuppermann\textsuperscript{b}

\textsuperscript{a}Smt Kanuri Santhamma Vitreoretina Center, L.V. Prasad Eye Institute, Hyderabad, India; \textsuperscript{b}UC Irvine Medical Center, Ophthalmology Clinic, Orange, Calif., USA

Abstract

Various pharmacologic vitreolysis agents, including hyaluronidase, urea, plasmin, dispase, tissue plasminogen activator and chondroitinase have been tested. Pharmacologic vitreolysis can avoid the complications of surgery such as cataract, endophthalmitis, retinal hemorrhage, tear or detachment, and anesthesia related complications. Hyaluronan is a major macromolecule of vitreous. It is a long, unbranched polymer of repeating disaccharide (glucuronic acid $\beta$ (1,3)-N-acetylglucosamine) moieties linked by $\beta$ 1–4 bonds. Hyaluronan is covalently linked to a protein core, to form a proteoglycan. It plays a pivotal role in stabilizing the vitreous gel. Hyaluronidase cleaves glycosidic bonds of hyaluronic acid and, to a variable degree, other acid mucopolysaccharides of the connective tissue. Dissolution of the hyaluronic acid and collagen complex results in decreased viscosity of the extracellular matrix. This in turn increases the diffusion rate of erythrocytes and exudates along with phagocytes through the vitreous and facilitates red blood cell lysis and phagocytosis.

Vitreous hemorrhage is a major cause of vision loss. The most common cause of vitreous hemorrhage is diabetic retinopathy, which can also lead to tractional retinal detachment \cite{1}. Enzymatic vitreolysis offers several advantages over conventional surgery, including the ability to treat the hemorrhage earlier, in an office setting, and with lower costs. Pharmacologic vitreolysis can also avoid the complications of surgery such as cataract, endophthalmitis, retinal hemorrhage, tear or detachment, and anesthesia related complications. A number of vitreolytic enzymes have been investigated, including hyaluronidase, plasmin dispase, tissue plasminogen activator, and chondroitinase, with varying effects. Such pharmacologic therapies rely upon a good understanding of the biochemical composition and organization of vitreous.

Ultrastructural studies have shown that the adult human vitreous contains fine, parallel fibers of collagen coursing in an anteroposterior direction \cite{2, 3}. Although several agents have been investigated in animal models, only a few have been employed in humans. Chondroitinase and hyaluronidase have been tested in clinical trials, but
Pharmacologic Vitreolysis

the former never entered phase II testing. Hyaluronidase (Vitrase) failed a phase III US Food and Drug Administration (FDA) trial conducted in the United States. The Vitrase clinical trial results will be discussed in more detail later in this chapter.

Hyaluronan

Hyaluronan is a major macromolecule of vitreous [3]. Hyaluronan is a long, unbranched polymer of repeating disaccharide (glucuronic acid β (1,3)-N-acetylglucosamine) moieties linked by β 1–4 bonds. It is a linear, left-handed, threefold helix with a rise per disaccharide on the helix axis of 0.98 nm. The sodium salt of hyaluronan has a molecular weight of 3 to 4.5 × 10^6 in normal human vitreous. Hyaluronan is not normally a free polymer in vivo, but it is covalently linked to a protein core, to form a proteoglycan. Hyaluronan plays a pivotal role in stabilizing the vitreous gel.

Collagen

Studies have shown that vitreous contains collagen type II, a hybrid of types V/XI, and type IX collagen in a molar ratio of 75:10:15, respectively [4]. Vitreous collagens are organized into fibrils with type V/XI residing in the core, type II collagen surrounding the core, and type IX collagen on the surface of the fibril. The fibrils are 7–28 nm in diameter, but their length in situ is unknown.

Supramolecular Organization

Vitreous is a dilute meshwork of collagen fibrils interspersed with extensive arrays of hyaluronan molecules. The collagen fibrils provide a scaffold-like structure that is ‘inflated’ by the hydrophilic hyaluronan. If collagen is removed, the remaining hyaluronan forms a viscous solution. However, if hyaluronan is removed, the gel shrinks but is not destroyed. Electrostatic binding occurs between the negatively charged hyaluronan and the positively charged collagen in vitreous [5].

Studies have shown that the chondroitin sulfate chains of type IX collagen bridge between adjacent collagen fibrils in a ladder-like configuration spacing them apart [6]. Such spacing is necessary for vitreous transparency, because keeping vitreous collagen fibrils separated by at least one wavelength of incident light minimizes light scattering, allowing the unhindered transmission of light to the retina for photoreception. Bishop [4] proposed that the leucine-rich repeat protein opticin is the predominant structural protein responsible for short-range spacing of collagen fibrils.

Numerous changes occur in the structure of vitreous with age. There is a significant decrease in the gel volume and an increase in the liquid volume of human vitreous.
Fig. 1. Studies in rabbit vitreous injected with Vitrase show digestion of hyaluronic acid and chondroitin sulfate compared to untreated controls.

Fig. 2. Clearing of dense vitreous hemorrhage of greater than one month duration in a patient with proliferative diabetic retinopathy. Schematic sequence above and photographic sequence below showing degree of vitreous hemorrhage immediately prior to injection of 55IU Vitrase, then one week post injection showing partial clearing, and finally two weeks post injection with near complete clearance of vitreous hemorrhage.
Derangement of the normal hyaluronan/collagen association results in the simultaneous formation of liquid vitreous and aggregation of collagen fibrils into bundles of parallel fibrils seen macroscopically as large fibers [7]. In the posterior vitreous, such age-related changes form large pockets of liquid vitreous.

Vitreoretinal adhesion at the posterior pole is more fascial than focal (at the disc, fovea, and along retinal blood vessels), consistent with the concept that the two tissues are held together by an extracellular matrix tissue [8]. With age, there is weakening of vitreoretinal adhesion, most likely due to biochemical alterations in the extracellular matrix tissue at the vitreoretinal interface. Studies using lectin probes have identified that one component of the extracellular matrix at the vitreoretinal interface [galactose-β (1,3)-N-acetylglucosamine] is present in youth but absent in adults [9]. This difference and others may play a role in the observed weakening of the vitreoretinal interface during aging.

**Anomalous PVD**

Anomalous PVD results when the extent of vitreous liquefaction exceeds the degree of vitreoretinal interface weakening, resulting in traction exerted at the vitreoretinal interface [10]. There can be various untoward effects of anomalous PVD. Effects upon the retina include hemorrhage, retinal tears and detachment, vitreomacular traction syndromes, and some cases of diffuse diabetic macular edema. Proliferative diabetic retinopathy can be greatly aggravated by anomalous PVD. Effects upon vitreous involve posterior vitreoschisis, where splitting of the posterior vitreous cortex and forward displacement of the vitreous body leave the outer layer of the split posterior vitreous cortex still attached to the retina. This can result in macular pucker, contribute to macular holes, or complicate proliferative diabetic retinopathy.

**Pharmacologic Vitreolysis**

Various pharmacologic vitreolysis agents, including hyaluronidase, urea, plasmin, dispase, tissue plasminogen activator and chondroitinase have been tested to date [11], but little is known about the exact mechanism of action of these substances, and so far none has met with sufficient success to result in widespread use.

*Hyaluronidase*

Hyaluronidase cleaves glycosidic bonds of hyaluronic acid and, to a variable degree, other acid mucopolysaccharides of the connective tissue. Dissolution of the hyaluronic acid and collagen complex results in decreased viscosity of the extracellular matrix. This in turn increases the diffusion rate of erythrocytes and exudates
along with phagocytes through the vitreous and facilitates red blood cell lysis and phagocytosis.

**Vitrase (Hyaluronidase)**

Vitrase was studied in a large, multicenter, randomized controlled trial [12]. A total of 1,125 patients with persistent vitreous hemorrhage were randomized to 55 IU (n = 365), 75 IU (n = 377), and saline (n = 383). The percentage of patients reaching primary efficacy by month 1 in the 55 IU, 75 IU, and saline groups was 13.2, 10.6, and 5.5% (p = 0.001 and p = 0.010, respectively). By month 3, 32.9, 30.5, and 25.6% of patients treated with 55 IU, 75 IU, and saline reached the primary efficacy endpoint (p = 0.025, p = 0.144, respectively). A statistically significant improvement in BCVA was seen. By month 3, 44.9 and 43.5% of patients in the 55 and 75 IU treatment groups had at least a 3-line improvement in BCVA compared with 34.5% of patients in the saline group (p = 0.004 and p = 0.011, respectively). The analysis of the investigator graded reduction in vitreous hemorrhage density also showed statistical significance in the 55 and 75 IU dose groups compared with saline by months 1, 2 and 3. In the analysis of the clinical assessment of therapeutic utility (clearance of the hemorrhage sufficient to diagnose the underlying pathology), there was a significant difference for patients in the 55 and 75 IU treatment groups compared with saline-treated patients by months 1, 2 and 3. By month 3, 40.8 and 39.3% of patients in the 55 IU (p = 0.001) and 75 IU (p = 0.002) groups, respectively, reached this endpoint compared with 28.5% of patients in the saline group.

In summary, data from the phase III clinical trials demonstrated that intravitreal injection of ovine hyaluronidase was efficacious and had a favorable safety profile. The primary ocular adverse event associated with the use of ovine hyaluronidase was acute, self-limited anterior uveitis. This iritis resolved with or without treatment, and without sequelae. Additionally, clinical experience has demonstrated that these injections do not prohibit or complicate subsequent vitrectomies when they are required.

**References**


Raja Narayanan, MD
Smt Kanuri Santhamma Vitreoretina Center, L.V. Prasad Eye Institute
L.V. Prasad Marg, Banjara Hills
Hyderabad 500034 (India)
E-Mail drnons@yahoo.com
Microplasmin-Assisted Vitrectomy

Arnd Gandorfer

Vitreoretinal and Pathology Unit, Augenklinik der Ludwig-Maximilians-Universität, Munich, Germany

Abstract

Microplasmin (Thrombogenics Ltd., Leuven, Belgium) is a recombinant molecule consisting of the catalytic domain of human plasmin. It is highly characterized, and supplied in a stabilized form, simplifying storage and administration. Microplasmin shares the same catalytic properties like human plasmin, but it is much more stable compared to plasmin. It has been shown previously that both plasmin and microplasmin are capable of inducing PVD. This chapter focuses on one of the most promising current concepts of pharmacologic vitreolysis, i.e. microplasmin-assisted vitrectomy. We report on the preclinical work of plasmin and microplasmin which lead to the clinical investigation of microplasmin in different clinical trials, the so-called MIVI trials.

Plasmin, a non-specific serine protease mediating the fibrinolytic process, acts also on a variety of glycoproteins including laminin and fibronectin. Both of them are present at the vitreoretinal interface, and they are believed to chemically bind the vitreous to the internal limiting membrane (ILM) of the retina [1–3].

In 1993, PVD could be achieved in rabbit eyes by intravitreal injection of the enzyme followed by vitrectomy [4]. In 1999, Hikichi et al. [5] confirmed complete PVD after injection of 1U plasmin and 0.5 ml SF₆ gas in the rabbit model, without evidence of retinal toxicity.

Unfortunately, plasmin is not yet available for clinical application, and alternative strategies have been pursued to administer the enzyme in vitreoretinal surgery. Tissue plasminogen activator was injected into the vitreous in an attempt to generate plasmin by intravitreal activation of endogenous plasminogen. In an animal model in rabbit eyes, complete PVD was observed in all eyes treated with 25 μg tissue plasminogen activator [6]. Breakdown of the blood-retinal barrier was necessary to allow plasminogen to enter the vitreous, and this was induced by cryocoagulation [6]. In

The author is co-founder of the Microplasmin Study Group and has a financial interest in pharmacologic vitreolysis.
two clinical pilot studies, 25 μg tissue plasminogen activator was injected into the vitreous of patients with proliferative diabetic retinopathy 15 min before vitrectomy [7, 8]. The results of both studies, however, were contradictory in terms of PVD induction and clinical benefit. Recently, Peyman's group [9] demonstrated PVD induction in rabbit eyes by an intravitreal administration of recombinant lysine-plasminogen and recombinant urokinase.

Autologous plasminogen purified from the patient's own plasma by affinity chromatography was converted to plasmin by streptokinase in vitro. 0.4 U of autologous plasmin enzyme (APE) was injected into the vitreous in patients with pediatric macular holes, diabetic retinopathy, and stage 3 idiopathic macular holes, followed by vitrectomy after 15 min [10–12]. All APE-treated eyes achieved spontaneous or easy removal of the posterior hyaloid including one eye that had vitreoschisis over areas of detached retina.

Microplasmin (Thrombogenics Ltd., Leuven, Belgium) is a recombinant product which contains the catalytic domain of human plasmin only [13]. It shares the catalytic properties of human plasmin, but is much more stable than the original molecule. Microplasmin is highly characterized. It is supplied in powder form which simplifies storage and administration.

As discussed in the first chapter, the goal of enzymatic vitreolysis is to manipulate the vitreous collagen pharmacologically, both centrally achieving liquefaction (synchisis), as well as along the vitreoretinal interface to induce posterior vitreous detachment (PVD, syneresis), and to create a cleavage plane more safely and cleaner than can currently be achieved by mechanical means [14–17].

**Preclinical Evaluation of Plasmin-Assisted Vitrectomy**

We investigated the effect of plasmin in porcine postmortem eyes and in human donor eyes. In porcine eyes, we observed a dose-dependent separation of the vitreous cortex from the ILM after intravitreal injection, without additional vitrectomy or gas injection [18]. In scanning electron microscopy, a bare ILM was achieved with 1 U of porcine plasmin 60 min after injection, and with 2 U of plasmin 30 and 60 min after injection, respectively. In control fellow eyes which were injected with BSS, the cortical vitreous remained attached to the retina [18]. For these experiments, we have developed a straightforward grading system that allows for easy and reliable quantification of cortical vitreous remnants at the ILM when scanning electron microscopy is performed [18].

In human donor eyes, 2 U of human plasmin from pooled plasma achieved complete PVD 30 min after injection, whereas the vitreoretinal surface of the fellow eyes was covered by collagen fibrils [19]. In both studies, transmission electron microscopy revealed a clean and perfectly preserved ILM in plasmin-treated eyes, and no evidence of inner retinal damage was seen [18, 19]. Li et al. [20] confirmed these
results, and reported a reduced immunoreactivity of the vitreoretinal interface for laminin and fibronectin following plasmin application.

In an experimental setting simulating the application of plasmin as an adjunct to vitrectomy, we injected human donor eyes with 1 U of plasmin, followed by vitrectomy 30 min thereafter [21]. All plasmin-treated eyes showed complete PVD, whereas the control eyes which were vitrectomized conventionally had various amounts of the cortical vitreous still present at the vitreoretinal interface [21].

**Preclinical Evaluation of Microplasmin-Assisted Vitrectomy**

In 2001, we approached Thrombogenics, a drug development company which had manufactured recombinant microplasmin for clinical investigation in patients with stroke and peripheral artery occlusive disease. Given our preclinical work with human plasmin from pooled plasma, we tested microplasmin in human postmortem eyes in the same manner as we did before with human plasmin. There was a clear dose-response relationship of microplasmin comparable to that of human plasmin. Complete vitreoretinal separation was possible without affecting retinal morphology [22]. We went on testing the substance in the cat model in vivo (fig. 1). No alteration in retinal ultrastructure was seen, and there was no change in the antigenicity of neurons and glial cells [22]. This was important to know as Mueller cells are very sensitive to any form of ocular trauma and intraocular surgery. Further animal studies conducted by the manufacturer followed in different species. Formal toxicology testing was performed, and microplasmin entered the clinical phase.

**Clinical Evaluation of Microplasmin-Assisted Vitrectomy**

We designed the first clinical study investigating the effect of microplasmin in combination with vitrectomy (MIVI-I). This phase II study aimed at determining the safety of microplasmin following intravitreal injection. 60 patients were included, and all received an intravitreal injection of microplasmin before vitrectomy. In the six cohorts, either the dose or the incubation time between injection and surgery were increased. In this setting, microplasmin appeared to be safe, and preliminary efficacy results were promising.

There are several other European studies underway to investigate the effect of microplasmin on vitreolysis; a nonvitrectomy trial in patients with diabetic macular edema (MIVI-II), and a combined injection/vitrectomy trial in patients with traction maculopathy (MIVI-III). The results of the MIVI-I study have already served as a basis for the FDA opening study (MIVI-III) which was performed in the USA. According to a press release by Thrombogenics from June 2008, approximately 30% of patients showed relief of vitreoretinal traction without the need for vitrectomy.
Conclusion

Plasmin and microplasmin hold the promise of inducing complete PVD without causing morphologic alteration of the retina. Several independent studies confirmed a dose-dependent and complete vitreoretinal separation, associated with perfect preservation of the ultrastructure of the ILM and the retina [4, 5, 18, 19, 21, 22]. In addition, a dose-dependent liquefaction of the vitreous induced by microplasmin was demonstrated by dynamic light scattering in dissected porcine vitreous and in intact pig eyes, making plasmin and microplasmin the most promising agents for pharmacologic vitreolysis at the moment [23]. Data from the first clinical trial of microplasmin are available and are promising in terms of safety and efficacy. Randomized studies are now performed to further assess the safety and efficacy of microplasmin when used as an adjunct to vitrectomy, or even as its replacement.

References


Several enzymatic agents, such as autologous plasmin enzyme and recombinant microplasmin, are able to cause vitreous liquefaction and a complete posterior vitreous detachment (PVD). Advancements in research have helped to explain the complex interactions that occur in the vitreous cavity after a PVD is created. The development of a PVD is a dynamic process that is thought to have a larger impact on the vitreous cavity milieu than just a separation of the posterior cortical vitreous from the retina. Pharmacologic vitreodynamics attempts to explain the mechanical and biochemical changes that occur at the vitreoretinal junction after a PVD is formed. The flow of molecules into and out of the vitreous cavity and across the vitreoretinal junction is thought to be influenced by the presence or absence of a PVD. A microplasmin-induced PVD has been shown to alter the vitreous levels of several molecules, and a PVD may have a protective role in multiple diseases. Significant progress has been made in the field of pharmacologic vitreodynamics. As we improve our understanding of the molecular flux in the vitreous cavity, pharmacologic vitreodynamics will likely become more important as it may allow for improved manipulation of intravitreal molecules.

**Abstract**

Several enzymatic agents, such as autologous plasmin enzyme and recombinant microplasmin, are able to cause vitreous liquefaction and a complete posterior vitreous detachment (PVD). Advancements in research have helped to explain the complex interactions that occur in the vitreous cavity after a PVD is created. The development of a PVD is a dynamic process that is thought to have a larger impact on the vitreous cavity milieu than just a separation of the posterior cortical vitreous from the retina. Pharmacologic vitreodynamics attempts to explain the mechanical and biochemical changes that occur at the vitreoretinal junction after a PVD is formed. The flow of molecules into and out of the vitreous cavity and across the vitreoretinal junction is thought to be influenced by the presence or absence of a PVD. A microplasmin-induced PVD has been shown to alter the vitreous levels of several molecules, and a PVD may have a protective role in multiple diseases. Significant progress has been made in the field of pharmacologic vitreodynamics. As we improve our understanding of the molecular flux in the vitreous cavity, pharmacologic vitreodynamics will likely become more important as it may allow for improved manipulation of intravitreal molecules.

**Pharmacologic Vitreodynamics and Molecular Flux**

David T. Goldenberg\(^a\) • Michael T. Trese\(^a,b\)

\(^a\)Associated Retinal Consultants, Royal Oak, Mich., and \(^b\)Eye Research Institute, Oakland University, Rochester, Mich., USA

During the last 10 years, there has been substantial progress in our understanding of the vitreous and its role in disease pathogenesis. Manipulation of the vitreous with pharmacologic agents has improved our ability to define the complex relationships between the vitreous and the retina. Enzymatic agents such as autologous plasmin enzyme and recombinant microplasmin (ThromboGenics Ltd., Dublin, Ireland) are able to successfully cause vitreous liquefaction and a complete posterior vitreous detachment (PVD) [1–10]. Not only do these chemical agents cleave the vitreoretinal junction, but they may also help to explain some of the biochemical changes that occur in the vitreous cavity after a PVD is created. Pharmacologic vitreodynamics is a novel term that describes the use of enzymatic agents to manipulate the vitreous and,

Dr. Trese has a financial interest in plasmin and microplasmin.
more importantly, the complex mechanical and biochemical interactions at the vitreoretinal interface. Significant advancements have been made in the field of pharmacologic vitreodynamics, and this review will provide a summary of recent findings.

**Molecular Flux**

Drug injection studies into the vitreous cavity currently consider all vitreous cavities to be equal. In contrast, all vitreous cavities are not the same since vitreous liquefaction occurs to varying degrees in each eye. Furthermore, the posterior hyaloid may or may not be detached and the thickness and adherence of this posterior hyaloid may vary from location to location within an eye. It has been suggested that an attached posterior hyaloid (i.e. absence of a PVD) may influence the flux of molecules that cross the vitreoretinal junction [11]. It is possible that the posterior vitreous cortex creates a barrier to certain molecules in the vitreous cavity. For example, in hyaluronidase-injected cat eyes (vitreous liquefaction without a PVD) and in control eyes (no liquefaction and no PVD), the cortical vitreous appears more densely compressed at the retinal surface than in microplasmin-injected eyes which demonstrate a smooth retinal surface that is free of cortical vitreous (fig. 1) [8]. It is also possible that the connections between the vitreous and retina provide a barrier to molecular diffusion. Laminin and fibronectin are the main glycoproteins that bind vitreous collagen fibers of the posterior vitreous cortex to the internal limiting membrane (ILM) [12]. The failure of tissue plasminogen activator (tPA) to penetrate the retina has been attributed to its specific binding to fibronectin, in addition to other proteins at the vitreoretinal juncture [13]. Plasmin enzyme and microplasmin are able to hydrolyze laminin and fibronectin at the vitreoretinal junction [14, 15], and studies are currently underway to determine if a microplasmin-induced PVD increases retinal penetration of tPA. The factors that contribute to the flux of molecules into and out of the vitreous cavity are likely to be multifactorial. Nevertheless, multiple studies suggest that a PVD may play a role in this process.

**PVD and Vitreous Oxygen Levels**

In the nonvitrectomized eye, there is a concentration gradient of oxygen from the retina into the vitreous gel [16]. Previous reports have shown that oxygen levels in the vitreous cavity increase after pars plana vitrectomy, and this increase is sustained for several months after surgery [16, 17]. It has been suggested that the sustained increase in oxygen levels is due to the circulation of fluid in the vitreous cavity that occurs after removal of the vitreous gel [17]. Sebag et al. [9] have shown that injection of microplasmin increases the vitreous diffusion coefficient in porcine eyes using dynamic light scattering. Their results indicate increased liquefaction of the vitreous...
following microplasmin injection [9], which may allow for increased mixing and oxygen exchange within the vitreous cavity. Recently, Quiram et al. [8] used animal models to study the effect of an enzymatic-assisted PVD on the oxygen concentration in the vitreous. The authors showed that oxygen flow across the vitreoretinal interface can be altered by a microplasmin-induced PVD [8]. Specifically, they found that a microplasmin-induced PVD increases vitreous oxygen levels and the rate of oxygen exchange within the vitreous cavity [8]. This effect was not seen with hyaluronidase and liquefaction of the vitreous cavity alone [8], which suggests that oxygen diffusion may be more influenced by a PVD rather than vitreous liquefaction. Even then, the authors were unable to determine if the elevated vitreal oxygen was due to an increase in oxygen diffusion or an increase in bulk flow (mixing) of oxygen in liquefied vitreous.

In patients with proliferative diabetic retinopathy (PDR), retinal ischemia leads to elevated levels of vascular endothelial growth factor (VEGF) which induces retinal neovascularization and macular edema [18, 19]. Pan-VEGF blockade with intravitreal bevacizumab results in regression of retinal neovascularization and reduction of fluorescein angiography leakage [20]. It has been proposed that PVD formation with microplasmin could increase vitreous oxygen levels, thereby reducing the ischemic drive to produce VEGF with subsequent reduction in neovascularization [8]. Consistent with this theory is the observation that vitrectomy with complete posterior vitreous separation is believed to reduce the progression of diabetic retinopathy and macula edema in diabetic eyes [3, 21, 22]. Furthermore, a complete PVD has

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**Fig. 1.** Scanning electron microscopy of cat vitreoretinal interface. **a** Control showing surface with dense network of cortical vitreous. **b** After hyaluronidase injection, condensed cortical vitreous is present at the vitreoretinal interface following vitreous liquefaction without posterior vitreous separation. **c** Smooth retinal surface free of cortical vitreous following induction of a posterior vitreous detachment with microplasmin. Originally published in Retina 2007; 27: 1090-1096. Reprinted with permission from the publisher.
been found to be a strong negative risk factor for the progression of diabetic retinopathy [21].

**PVD and Other Vitreous Molecules**

The change in vitreous oxygen molecular flux that was demonstrated by Quiram et al. [8] may be used as a marker for the behavior of other molecules in the extracellular matrix of the vitreous cortex. Preliminary data from cat models suggest a microplasmin-induced PVD leads to decreased levels of VEGF in the vitreous [23]. In addition, we have shown that the retinal penetration of intravitreal bevacizumab is initially increased in rabbits with a microplasmin-induced PVD when compared with control eyes [24]. It is possible that a PVD may influence the concentration of many molecules and growth factors in the vitreous cavity [11]. It is unclear whether a specific relationship exists between a PVD and vitreous growth factors. Prospective clinical trials will be needed to address this theory.

Pharmacologic vitreodynamics may also have a protective role in age-related macular degeneration (AMD). A higher incidence of attached posterior vitreous has been reported to occur in patients with all types of AMD (exudative and nonexudative) [25]. Additional studies have concluded that persistent attachment of the posterior vitreous cortex to the macula may be a risk factor for the development of exudative AMD [26, 27]. Krebs et al. [26] attributed these findings to several theories: (1) chronic inflammation in the macula due to persistent vitreomacular adhesion, (2) the presence of an attached posterior vitreous prevents the normal diffusion of oxygen and other nutrients across the vitreomacular interface, and (3) the presence of an attached posterior vitreous confines VEGF and other cytokines in the macula. The association between vitreomacular adhesion and exudative AMD is probably multifactorial. However, if the diffusion of molecules across the vitreoretinal junction plays a role, then pharmacologic vitreodynamics has the potential to significantly alter the natural history of AMD.

**Conclusion**

The emerging field of pharmacologic vitreodynamics presents a new frontier in the management of vitreoretinal diseases. As we improve our understanding of the molecular flux in the vitreous cavity, pharmacologic vitreodynamics will likely become more important as it may allow for improved manipulation of intravitreal molecules. The use of enzymatic agents may soon enable physicians to better tailor their treatments for a variety of vitreoretinal diseases. Pharmacologic alteration of the vitreous is an evolving modality that will likely be used more frequently for therapy and preventative measures.
References


Glial Cell Changes of the Human Retina in Proliferative Vitreoretinopathy

Louisa Wickham · David G. Charteris

Vitreoretinal Unit, Moorfields Eye Hospital, London, UK

Abstract

Retinal detachment initiates a complex series of cellular and molecular changes. Because of the difficulty in obtaining retinal tissue from patients with retinal detachments, most investigations of the cellular changes following retinal detachment have been carried out on animal models. Animal experiments suggest that the cellular response to retinal detachment may be broadly described as; partial dedifferentiation of retinal pigment epithelium cells, proliferation and migration of retinal pigment epithelium cells into the subretinal space, degeneration of the photoreceptor outer segments and synaptic terminals, photoreceptor cell death, structural remodelling of second-order and third-order retinal neurons, proliferation of all non-neuronal cell types within the retina and Müller cell hypertrophy leading to glial scar formation. Each of these stages are considered in turn and related to findings in human proliferative vitreoretinopathy.

Separation of neural retina from the retinal pigment epithelium (RPE) in retinal detachment initiates a complex series of cellular and molecular changes. Evidence that this process might be arrested or reversed following re-apposition of the neural retina with the RPE comes from clinical studies that show good visual outcomes following retinal detachment surgery [1, 2]. However, in some patients the visual outcomes are very poor, particularly in those who develop proliferative vitreoretinopathy (PVR) [3–6], suggesting that remodelling initiated by detachment may continue despite restoration of retinal anatomy. An understanding of cellular changes following retinal detachment together with the wound healing response seen following reattachment is essential in the identification of adjuncts aimed at modifying the development of PVR.

Because of the difficulty in obtaining retinal tissue from patients with retinal detachments, most investigations of the cellular changes following retinal detachment have been carried out on animal models, particularly cat and rabbit. The feline retina
is rod dominant, has an intraretinal circulation that is excluded from the photoreceptor layer and a choroidal circulation that supplies the photoreceptor layer. The rabbit retina is also rod dominant but has no intraretinal vasculature with the inner retina being supplied by vessels that lie on the vitreal surface. The rabbit retina is more difficult to conduct experiments on as the rabbit eye is a smaller and the retina tends to degenerate very rapidly following detachment. The study of animal retinal detachment through controlled experiments has provided insight into the cellular effects of different stages of retinal detachment. Broadly these may be described as [7]:

1. Partial dedifferentiation of RPE cells.
2. Proliferation and migration of RPE cells into the subretinal space.
3. Degeneration of the photoreceptor outer segments and synaptic terminals.
4. Photoreceptor cell death.
5. Structural remodelling of second-order and third-order retinal neurons.
6. Proliferation of all non-neuronal cell types within the retina.
7. Müller cell hypertrophy leading to glial scar formation.

**Partial Dedifferentiation of RPE Cells**

The RPE is a polarised monolayer of neuroepithelial cells that rest on Bruch’s membrane [8]. Within 24 h of retinal detachment the long microvilli (5–15 μM) that usually ensheath the photoreceptors are replaced by a fringe of microplicae (~5 μM in length) [9]. Within 1–3 days the usually smooth surface of the RPE becomes mounded with protrusions of cytoplasm into the subretinal space and the nucleus becomes displaced to a more apical position.

**Proliferation and Migration of RPE Cells into the Subretinal Space**

In the feline model, experiments using $^3$H-thymidine have shown that within 72 h of retinal detachment the RPE has begun to proliferate and may be observed as areas of hyperplasia within the RPE monolayer, particularly at the junction of attached and detached retina [9, 10]. RPE proliferation is confined to areas of retinal detachment suggesting that the interaction between RPE and neural retinal has an antimitotic effect [9]. RPE proliferation and reversal of cellular polarity may be responsible for affecting regeneration of photoreceptor outer segments following retinal reattachment [11]. In reattached cat retina, areas where the apposition of the retina with the apical surface of the RPE was retained and photoreceptors were quick to regenerate; however, in areas where RPE and Müller cells had proliferated into the sub-retinal space, photoreceptor recovery was poor or nonexistent [12]. Further, to establish a functional relationship between photoreceptors and the RPE, the interaction between the apical surface of the RPE and photoreceptor outer segments must be restored to
allow the transport of retinoids vital for the visual cycle [7]. RPE cells with rounded apical surfaces or cells with reversed polarity bear little resemblance to normal RPE and this is likely to influence photoreceptor recovery.

The subretinal space is usually free of cells; however, within 24 h of retinal detachment a number of cell types, e.g. polymorphonuclear neutrophils and monocytes, migrate into this space from the choroidal and retinal capillaries [9]. Free RPE cells are also seen in the subretinal space within 72 h of retinal detachment and frequently contain outer segment fragments, indicating that they may play a role in phagocytosis of cellular debris [9, 13, 14].

Degeneration of the Photoreceptor Outer Segments and Synaptic Terminals

Within 12 h of experimental retinal detachment, photoreceptor outer segments show evidence of structural damage [9]. Initially, the distal end of the outer segment becomes vacuolated or distorted and by 24–72 h all rod and cone outer segments are significantly shorter and distorted with disorientated discs [15]. Outer segment debris is shed into the subretinal space where it is phagocytosed by RPE cells and macrophages which have migrated into the area.

Although retinal detachment interrupts the process of disc production and shedding, outer segment specific proteins continue to be produced but localise to abnormal cellular locations. Opsin, normally concentrated in the outer segment, begins to accumulate in the plasma membrane vitread to the outer segment within a day of experimental retinal detachment (fig. 2) [15]. Peripherin/rds, another outer segment protein specific to the disc rims, is also redistributed and begins to appear in cytoplasmic vesicles [16]. Cone outer segment proteins appear to be more susceptible to damage, with redistributed cone opsins persisting for just 1 week following retinal detachment, after which they are only observed in occasional cells [17].

Inner segments of photoreceptors begin to show signs of degeneration 1–3 days following retinal detachment such as swelling, disruption and loss of mitochondria, disruption of the rough endoplasmic reticulum and Golgi apparatus and an overall reduction in size [7]. The connecting cilium, a structure essential for the production of the outer segment should retinal reattachment occur, is retained even in severely affected inner segments [9].

Photoreceptor Cell Death

In the feline model, photoreceptor cell bodies and synaptic terminals show extensive vacuolisation, degeneration of mitochondria and disorganisation of the microtubules and actin filaments [7]. Degeneration of photoreceptors is variable and areas of extensive degeneration coexist with areas of relatively intact photoreceptors [15].
Cook et al. [18] demonstrated that cell death via the apoptotic pathway peaks three days following retinal detachment and then continues at low levels for as long as the retina is detached. More recently, photoreceptor apoptosis has also been demonstrated in human retinal specimens, with a peak at 2 days following retinal detachment [19]. In the feline model, approximately 80% of the photoreceptors are lost within 90 days following retinal detachment [20]. Rod cell bodies appear to degenerate quicker than...
cones following retinal detachment [21], and following cell death photoreceptors are extruded into the subretinal space where they are phagocytosed.

**Structural Remodelling of Second-Order and Third-Order Retinal Neurons**

Photoreceptor cell bodies extend processes into the outer plexiform layer where they form synapses with second order neurones. Rods and cones have characteristic synaptic terminals called spherules and pedicles respectively. Synaptophysin labelling 3 days following retinal detachment shows many synaptic terminals retracted towards the photoreceptor cell body, with migration of some synaptic structures into the outer nuclear layer from the outer plexiform layer [22]. In the same way that cones appear to degenerate slower than rods, cone pedicles appear to survive longer following retinal detachment than spherules [20].

Changes in the synaptic terminals are accompanied by the growth of processes from the rod bipolar cells and horizontal cells into the outer nuclear layer [22]. Ganglion cells also become reactive, and begin to re-express GAP 43, a protein expressed early in cell body development for the formation of synaptic connections between ganglion cell axons and the brain [23, 24]. Horizontal and ganglion cells show dramatic and extensive remodelling, growing neurites that appear to be attracted to Müller cells and extend into scars both in the subretinal space and on the vitreal surface [23, 25]. By contrast, rod bipolar cell dendrite growth is less aggressive, being targeted towards the retracted terminals of the rod photoreceptors.

**Proliferation of Non-Neuronal Cell Types**

Within 24 h of retinal detachment all non-neuronal cell types (astrocytes, Müller cells, pericytes, capillary endothelial cells and microglia) display signs of proliferation [26, 27]. Cellular proliferation appears to peak within 3–4 days of retinal detachment after which it slowly reverts to normal levels over a number of weeks [26, 27].

**Müller Cell Hypertrophy Leading to Glial Scar Formation**

Changes in the Müller cell are seen within 1 day of retinal detachment, including changes in protein expression and early growth of Müller cell processes [28]. Within 3 days, Müller cell bodies have migrated to the outer nuclear and outer plexiform layers into the spaces left by dying photoreceptors, and processes begin to extend into the subretinal space through localised disruptions in the outer limiting membrane. Following retinal detachment, Müller cell processes within the retina show greatly increased expression of both vimentin and glial fibrillary acidic protein (GFAP),
whereas segments of the Müller cell processes that extend through the outer limiting membrane into the subretinal space preferentially express vimentin [28, 29]. These processes appear to penetrate the outer limiting membrane adjacent to cone photoreceptors, often growing for long distances on the photoreceptor border and later forming multi-layered scars [28]. Following retinal reattachment, Müller cell processes within the subretinal space appear to inhibit the regeneration of photoreceptors and may therefore be influential in determining visual recovery [30].

**Retinal Recovery and the Development of PVR following Re-attachment**

Retinal recovery following retinal attachment relies on re-establishing the cell-to-cell contact of RPE cells and photoreceptors. This involves redifferentiation of the RPE apical surface, re-ensheathment of the regenerating outer segments, and probably resynthesis of inter-photoreceptor matrix components. In the feline model, retinal reattachment within 1–3 days is very effective at reversing the cellular changes induced by retinal detachment [9, 31]. Following reattachment outer segments recover to approximately 70% of their length, photoreceptor apoptosis is arrested and cellular proliferation is reduced [32]. Restoration of retinal anatomy following detachment is not uniform. The morphological appearance has been described as a ‘patchwork’, with areas of variability in outer segment length and levels of protein expression both within areas of retina and between different retinas [15, 32].

In areas where Müller cell hypertrophy has led to the formation of a subretinal scar, photoreceptors only show limited signs of recovery with no outer segment regeneration [30]. Moreover, reattachment appears to induce growth of Müller cell processes into the vitreous which form epiretinal membranes and act as a substrate for ganglion cell growth and neurite extension and also appear to induce growth of rod axons into the inner retina [28, 30].

**Cellular Changes in Human PVR**

Human correlates of animal experiments exist. Retinal tissue removed from post mortem specimens and from patients undergoing retinal detachment surgery has demonstrated changes similar to those seen in animal models [33–36]. In animal studies, retinal detachments are induced in a controlled manner using a micropipette to inject fluid between the neural retina and RPE. This differs from human studies where the precise onset of retinal detachment can be difficult to determine and may vary within the eye according to its rate of progression. In addition, the process of acute retinal tear formation can result in the liberation of mitotically active RPE cells into the subretinal and preretinal spaces. These RPE cells may subsequently contribute to the formation of complex epiretinal membranes seen in human PVR [37]. A case report
of an acute retinal detachment showed that retinal tissue adjacent to the retinal tear demonstrated more advanced degenerative changes when compared with that from areas more distant from the breaks [36].

When retinal detachment is complicated by PVR samples obtained from patients undergoing retinectomy provide an insight into the pathology in more chronic stages of retinal detachment. In a study of 16 retinectomy specimens taken from patients with PVR, Sethi et al. [34] demonstrated that the response to prolonged retinal detachment with PVR in humans was similar to that observed in chronic detachment animal models. In humans, as in animal models, photoreceptors were degenerate and intracellular redistribution of opsin proteins to the plasma membrane was observed. In cones, labelling with anti M/L cone opsin showed degenerate outer segments and faint staining of swollen inner segments and in severe PVR staining of cone opsins was absent. Rod synaptic terminals showed remodelling with extension of rod bipolar cell dendrites and horizontal cell processes into the outer nuclear layer.

There was also upregulation of neurofilament and growth-associated protein-43 (GAP-43) expression in large ganglion cells with neurite sprouting. All retinectomy specimens showed a marked upregulation of Müller cell and astrocyte expression of GFAP and vimentin with areas of increased glial tissue replacing degenerated retinal neurons [34]. In some sections Müller cells breached the ILM and extended onto the retinal surface where they formed a component of epiretinal membrane [38]. The presence of neurites in sub- and epiretinal membranes has also been demonstrated in retinal specimens taken at the time of PVR surgery [39].

Close correlation of human and animal models of retinal detachment support the use of animal models in the characterisation of glial cell changes in PVR. Further, animal models are a valuable resource allowing investigation of the effects of potential adjuncts in this complex process.

References


Alkylphosphocholines: A New Approach to Inhibit Cell Proliferation in Proliferative Vitreoretinopathy

Kirsten H. Eibl\textsuperscript{a} · Steven K. Fisher\textsuperscript{b} · Geoffrey P. Lewis\textsuperscript{b}

\textsuperscript{a}University Eye Hospital, Ludwig-Maximilians-University, Munich, Germany; \textsuperscript{b}Neuroscience Research Institute, University of California, Santa Barbara, Calif., USA

Abstract

Proliferative vitreoretinopathy represents the major complication in retinal detachment surgery and occurs in about 5–15% of cases resulting in a significant loss of vision despite multiple surgical procedures. Although successful anatomical reattachment is usually achieved, the reduction in central vision often remains permanent due to the intraretinal changes induced by retinal detachment and the subsequent proliferative response within the retina. Retinal Müller glial cells play a pivotal role in this process together with retinal pigment epithelial cells which are dispersed in the vitreous and stimulated by growth factors and serum in the vitreous after the breakdown of the blood-retinal barrier.

Alkylphosphocholines (APCs) are effective inhibitors of human ocular cell proliferation and currently in clinical use for the treatment of protozoan and malignant diseases. Previously, we have demonstrated that APCs can inhibit human retinal Müller glial cell proliferation and attenuate the hypoxia-induced actin filament expression in these cells. Moreover, retinal pigment epithelial (RPE) cell spreading and migration as well as proliferation and cell-mediated membrane contraction are effectively inhibited by APCs at nontoxic concentrations in vitro. The first in vivo toxicity studies in the rat eye also did not display any toxic effect of APCs to retinal tissue 7 days after intravitreal administration. Finally, in a well-established in vivo model for PVR, we were able to demonstrate that APCs can dramatically reduce intraretinal Müller glial cell proliferation induced by experimental retinal detachment in nontoxic concentrations. Thus, APCs are promising, novel pharmacologic substances that may be a useful adjunct to reattachment surgery by reducing the intraretinal proliferation induced by detachment and hence reduce the incidence of PVR.
Cell Biology of Retinal Detachment and Proliferative Vitreoretinopathy

Following retinal detachment, a cascade of events are set in motion: photoreceptor outer segments degenerate, some photoreceptors die by way of apoptosis [1] and synaptic terminals undergo significant remodeling. In response to changes in photoreceptors, in particular the loss of synaptic connections, second-order neurons extend neurites into the outer retina [2]. Ultimately, the ganglion cells respond to detachment by also elaborating new processes throughout the retina [3]. While loss of photoreceptors and ‘downstream’ neuronal changes most certainly contribute to a loss in visual acuity, non-neuronal cells, in particular the glial cells, are also thought to play a role in the continued reduction in vision following retinal reattachment surgery and contribute to the subsequent proliferative response that occurs within the retina [4, 5]. Following experimental retinal detachment, it has been shown that all non-neuronal cell types are stimulated to divide [6, 7]. These cell types include Müller cells, astrocytes, RPE cells, microglia and macrophages; however, the Müller glial cell appears to be the cell type undergoing the highest level of cell division. Indeed, Müller cell proliferation has been implicated in the pathogenesis of various human retinal diseases including retinal degeneration [8], retinal detachment and PVR [9], proliferative diabetic retinopathy (PDR) [10] and epiretinal membrane formation in idiopathic surface wrinkling maculopathy [11–13]. Moreover, Müller cells are thought to play a role in the degenerative events occurring in attached retinal regions within the detached eye [5, 14].

Intraretinal glial cell proliferation is one of the earliest responses to retinal detachment [6, 7]. It is accompanied by rapid glial hypertrophy and upregulation of intermediate filament proteins [15]. These events most likely contribute to the growth of these cells onto either the sub- or epiretinal surface. Indeed, Müller cells have been identified in both human retinal tissue [9, 11–13] and experimental animal models of retinal detachment [6, 8]. Importantly, the presence of these glial ‘membranes’ can have devastating consequences for the return of good vision. With subretinal growth, termed subretinal fibrosis, glial cells can prevent the regrowth of photoreceptor outer segments following reattachment surgery [16] while epiretinal growth can cause re-detachment of the retina [17]. The fact that these events occur rapidly soon after detachment indicates that treatment with antiproliferative agents should also begin at this time. In addition, since it has been shown that proliferation continues following retinal reattachment, albeit at low levels [18], successful inhibition of membrane formation may require not only early but also long-term delivery of the agent.

The stimulus for initiating the proliferation of cells is unknown; however, due to the breakdown of the blood-retinal barrier after retinal detachment, there is certainly an increased amount of serum and growth factors present in the vitreous cavity that may promote proliferation of Müller glial and RPE cells [19, 20]. Indeed, intravitreal injection of basic fibroblast growth factor into a normal feline eye, has been shown to induce proliferation of non-neuronal cells, upregulate intermediate filament proteins, and stimulate the formation of epiretinal membranes [21].
Another factor that is most certainly involved in the response of the retina following detachment is hypoxia of the outer retina as the neural retina becomes distanced from the choriocapillaris. Hypoxia is known to cause significant changes in the architecture of the actin cytoskeleton and thus plays a pivotal role in complex cellular events like migration and proliferation [22]. Moreover, treatment of animals with retinal detachment by hyperoxia (70% inhaled O₂) results in reduced photoreceptor deconstruction and fewer dividing Müller cells [23]. Finally, while PVR is generally considered a ‘disease’ of proliferation, cellular hypertrophy is also most certainly involved. Thus, the crucial pathophysiologic events to be influenced by a novel intravitreal pharmacotherapy are first, the serum/growth factor-induced proliferation/hypertrophy of Müller glial and RPE cells, and second the hypoxia-induced glial changes with deconstruction of photoreceptors.

**Current Therapeutic Concepts**

Surgical intervention in itself is insufficient to control the cellular responses of PVR and, therefore, a pharmacological concept is warranted as an adjuvant therapy in combination with complex retinal reattachment surgery. Current pharmacologic intervention focuses primarily on antiproliferative and anti-inflammatory agents to prevent PVR [24]. A number of antiproliferative pharmacologic substances such as colchicine, daunomycin and 5-fluouracil have been tested for their effects on human retinal glial cells in vitro [25]. However, these substances are currently not part of any routine clinical treatment for PVR. One of the most promising candidates, 5-fluouracil, combined with low-molecular heparin, has recently been tested in a large clinical trial; however, it proved to be ineffective in reducing the incidence of PVR [26, 27]. A number of factors most likely contribute to the failure of studies such as these, one of which certainly involves choosing the optimal treatment regime. Based on animal studies, the ideal treatment would involve early administration of the agent, perhaps at the time of reattachment surgery in high-risk cases, combined with sustained delivery without causing toxicity to the retina. We feel that alkylphosphocholines (APCs), encapsulated in liposomes meet these criteria.

**Alkylphosphocholines**

Alkylphosphocholines (APCs) are synthetic phospholipid derivatives (fig. 1) and known as effective inhibitors of cellular proliferation. Clinically, they are currently applied for the treatment of protozoan [28] and malignant diseases [29]. In addition, APCs have been shown to inhibit human ocular cell proliferation which may have therapeutic implications for a number of proliferative ocular conditions including
those secondary to retinal detachment [30] as well as the scar formation of Tenon fibroblasts after glaucoma filtration surgery [31].

Their mechanism of action involves binding to the membrane-bound G-protein PKC (protein kinase C) which is part of a major intracellular second-messenger systems that regulates cell attachment, spreading, migration and proliferation. In ocular cells (Tenon fibroblasts and retinal pigment epithelial cells) and in the leukemia cell line HL60, APCs are effective inhibitors of PKC [31–33]. Also, PKC has been shown to be associated with a downregulation of focal adhesion kinase (FAK) [34]. FAK is one of the key cytoplasmic tyrosine kinases and acts as a potential integrin effector. A high proliferation rate is associated with an increased FAK activity in malignant glioma cells [35]. Moreover, FAK directs signaling molecules like phosphatidylinositol 3-kinase-Akt/PKB (PI3K) to focal contacts between cells and decides if cells migrate or proliferate [36]. Since APCs have been reported to inhibit the PI3K pathway in epithelial carcinoma cell lines [37], actin organization and focal contact dynamics which are in part determined by a PKC [38] and a FAK-directed signaling cascade might be influenced by APCs via both pathways.

Previously, we have been able to demonstrate that APCs can inhibit RPE cell spreading and migration [39] as well as proliferation and cell-mediated membrane contraction [32] at nontoxic concentrations in vitro. Moreover, the first in vivo toxicity studies in the rat eye did not display any toxic effect of APCs to retinal tissue 7

![Fig. 1. Chemical structure of the alkylphosphocholines oleyl-phosphocholine (C18:1-PC, a), (Z)-10-eicosenyl-phosphocholine (C20:1-PC, b), (Z)-12-heneicosenyl-phosphocholine (C21:1-PC, c), and erucyl-phosphocholine (C22:1-PC, d) applied in ophthalmic research.](image-url)
days after intravitreal administration [40]. Recent in vitro [41] and in vivo [30] studies examining the effect of alkylphosphocholines on retinal Müller glial cells have also been performed to further elucidate their potential therapeutic role in retinal proliferative diseases. Thus, APCs are promising, novel pharmacologic substances which have been found to be effective in controlling both the Müller cell [36] and RPE-related component of PVR pathogenesis [32, 34].

**In Vitro Effects**

To examine the in vitro effects of APCs on retinal Müller glial cells, primary Müller glial cells were prepared from Long-Evans rats in accordance with applicable German laws and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Isolated cells were routinely evaluated by immunofluorescence microscopy using a monoclonal antibody against vimentin. Also, the spontaneously immortalized human Müller cell line, MIO-M1, was used to evaluate the role of the alkylphosphocholines oleyl-phosphocholine (C18:1-PC; fig. 1a) and erucyl-phosphocholine (C22:1-PC; fig. 1d) on cell proliferation and F-actin stress fiber expression under normoxic and hypoxic conditions. To determine the levels of Müller glial cell proliferation, the incorporation of 5-bromo-2-deoxyuridine (BrdU) was measured after assessment of toxicity by the trypan blue exclusion assay. For detection of F-actin stress fibers, the rhodamine-phalloidin staining protocol was applied.

With this in vitro study, we could demonstrate that C18:1-PC and C22:1-PC are able to inhibit the proliferation of primary rat retinal Müller glial cells (fig. 2a) as well as of the immortalized human retinal Müller glial cell line MIO-M1 (fig. 2b) in a dose-dependent manner. Effective concentrations were non-toxic and able to attenuate the content of F-actin stress fibers in these cells which were induced by hypoxia, a key feature in the pathogenesis of retinal diseases. Therefore, in conclusion, this study provides further evidence that APCs might offer a novel treatment strategy for the prevention of PVR: first, they inhibit Müller glial cell proliferation and second, they are able to diminish the hypoxia-related upregulation of F-actin stress fibers in vitro [41].

**In Vivo Studies**

A subsequent evaluation of the results obtained in vitro has been performed in a well established in vivo model for PVR, the rabbit eye, where APCs have been found to be effective inhibitors of intraretinal proliferation after experimental retinal detachment [30].

Retinal detachments were created in adult New Zealand Red pigmented rabbits by infusing a solution of sodium hyaluronate (0.25% in balanced salt solution; BSS)
via a glass pipette between the neural retina and RPE. Two experimental conditions were tested: (1) whether APC treatment was more effective given at day 1 or day 2 after detachment, and (2) whether liposome-bound APC was more effective than the free drug. Control eyes were injected with balanced salt solution on day 1. In treated eyes, 100 μm of APC in 50 μl BSS was injected intravitreally via a 30-gauge needle. On day 3 after detachment, the animals were injected with 10 μg BrdU. The eyes were bisected and half of each eye, containing both attached and detached regions, was fixed in 4% paraformaldehyde for immunocytochemical analysis. Anti-BrdU was used to detect dividing cells, antivimentin to determine the extent of Müller cell hypertrophy and isolectin B4, *Griffonia simplicifolia* to label microglia and macrophages. The other half was placed in 1% paraformaldehyde plus 1% glutaraldehyde in phosphate buffer for light and electron microscopic analysis to determine potential toxic effects of APC. All experimental procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Animal Resource Center of the University of California, Santa Barbara, Calif., USA.

In control, nondetached retina, no anti-BrdU-labeled cells were detected (fig. 3a). In control detachments, Müller cells represented the largest subgroup of BrdU-labeled cells (fig. 3b, red) contributing to the massive intraretinal scar formation. The isolectin B4-labeled microglia within the retina formed the second largest subgroup and the isolectin B4-labeled macrophages in the subretinal space formed the third (fig 3a–e, both cell types in blue). In the APC injected eyes, the number of

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**Fig. 2.** C18:1-PC and C22:1-PC inhibit proliferation of retinal Müller glial cells. Primary rat Müller glial cells (a) or MIO-M1 cells (b) were cultured in 96-well microplates and incubated with different concentrations of the APCs. Proliferation of cells was assessed by measurement of BrdU incorporation (p < 0.05 for all concentrations >25 nM).
Fig. 3. Laser scanning confocal images of retinal sections labeled with probes to vimentin (green), BrdU (red) and isoelectin B4 (blue). a Undetached control retina. Anti-vimentin labeled Müller cells extend from the ganglion cell layer (GCL) into the outer nuclear layer (ONL), and isoelectin B4 labeled fine microglial cell processes in the inner plexiform layer (IPL). b Detached control retina 3 days after detachment and saline injection on day 1. Anti-vimentin labeling increased in Müller cells to the outer limiting membrane (OLM), anti-BrdU labeled dividing cells primarily in the inner nuclear layer (INL), and isoelectin B4 labeled microglia that had rounded up and migrated throughout the retina. c Three days after retinal detachment and non-liposome-bound APCs injected on day 2. A slight reduction in the number of anti-BrdU-labeled Müller cells was observed; antivimentin and isoelectin B4 labeling appeared similar to the control. d, e Three days after retinal detachment and liposome bound drug injected on day 1. There was a significant decrease in anti-BrdU-labeled Müller cell nuclei although anti-vimentin and isoelectin B4 labeling appeared similar to controls. f RPE region, 3 days after retinal detachment and saline injection on day 1. A few anti-BrdU labeled cells were observed but this pattern was not significantly different from the drug-treated animals. Scale bars = 20 μm.
BrdU-labeled Müller cells was significantly reduced (fig. 3c–e) without effecting the number of microglia or macrophages. In both control and APC-treated animals, the anti-vimentin labeling increased in Müller cells to the outer limiting membrane and sometimes beyond indicating cellular hypertrophy in response to retinal detachment and hypoxia (fig. 3b, c). Anti-BrdU-labeled Müller cells were observed both in the inner nuclear layer (INL), their normal location in the retina, and in the ONL, as a result of migration into the outer retina (fig. 3b). Numerous anti-BrdU-labeled Müller cells and isolectin B4-positive cells were observed in retinas given free APC on day 2 (fig. 3c), whereas significantly fewer cells incorporated BrdU when the retinas were treated with the liposome-bound APC given on either day 1 (fig. 3d, e) or day 2. There was no evidence of toxicity detected in any of the treatment groups since the retinal morphology at the electron-microscopic level of treated animals appeared the same as in the control eyes [30]. The RPE was also stimulated to divide following detachment, as shown by BrdU incorporation (fig. 3f); however, the number of labeled cells was too low to detect a difference between treated and control animals.

Conclusions/Perspectives

In these recent in vitro and in vivo studies, we have been able to demonstrate the anti-proliferative effect of alkylphosphocholines on retinal Müller glial cells at nontoxic concentrations. By using a well-recognized animal model of experimental retinal detachment, we could show the safety and efficacy of APCs for inhibition of Müller cell proliferation after intravitreal injection in the rabbit. The effects on glial cell hypertrophy and expansion outside the retina, however, have yet to be determined. The in vitro assessment of F-actin stress fiber expression and distribution demonstrated a protective effect of APCs on human and rat Müller glial cells under hypoxic cell culture conditions.

The use of alkylphosphocholines may represent an important step towards the development of an adjuvant pharmacologic treatment strategy for PVR and clinical application of these compounds seems feasible. However, long-term toxicity studies in an in vivo model must be performed before APCs can be considered for clinical application.

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Kirsten H. Eibl, MD, FEBO
University Eye Hospital, Ludwig-Maximilians-University
Mathildenstrasse 8
DE-80336 Munich (Germany)
Tel. +49 89 5160 3800, Fax +49 89 5160 4788, E-Mail Kirsten.Eibl@med.uni-muenchen.de
Neuroprotection for Diabetic Retinopathy

Hisanori Imai · Ravi S.J. Singh · Patrice E. Fort · Thomas W. Gardner

Penn State Hershey Eye Center and Department of Cellular and Molecular Physiology,
Penn State College of Medicine, Hershey, Pa., USA

Abstract

Diabetic retinopathy (DR) is a neurodegenerative and microvascular disease resulting in functional and structural changes of all cell types in the retina. Several mechanisms for neuroretinal homeostasis, including the blood-retinal barrier, normal metabolite delivery into the retina, and the effect of neurotrophins for the retina, are impaired in DR. However, it is still not clear which components are most important for the development of DR and which may be most useful as therapeutic targets. In this chapter, we summarize the evidence for the neurodegeneration in DR and review normal mechanisms for maintenance of postmitotic cells in the retina and alterations in normal maintenance pathways in DR with emphasis on ‘neuroprotection’. Finally, we discuss current neuroprotective strategies and future directions for the treatment of DR.

Until recently, the concept of ‘neuroprotection’ for diabetic retinopathy (DR) would have seemed implausible given that the view of the disease as a purely microvascular problem prevailed. We currently consider DR as a ‘neurovascular’ disorder [1] since the retina is part of the nervous system, because neurons are the cells that subserve visual function and must by definition be altered when vision is lost, as in DR.

In this review, we emphasize an integrated view of the retina (a ‘network’) in which the blood-retinal barrier (BRB) is vital for neural retinal function and vice versa. Therefore, maintenance of microvascular integrity may be considered to be neuroprotective as much as strategies that target neurons or glial cells directly. Likewise, regulation of normal anabolic and synthetic processes, such as glucose metabolism, and lipid, nucleic acid and protein synthesis is ‘neurotrophic’ as much as the actions of peptide growth factors. Therefore, this review defines neuroprotection broadly and with the admission that we do not fully understand the mechanisms that maintain the retina in its highly differentiated state so it can perform its task of vision.
Evidence for Neurodegeneration in Diabetic Retinopathy

DR is a common complication of diabetes and approximately 75% of persons with type 1 diabetes develop retinopathy, while approximately 50% of persons with type 2 diabetes may develop retinopathy [2], and it is the leading cause of new cases of legal blindness in Americans between the ages of 20 and 74 years [3]. Neurodegeneration is recognized as a component of some retinal diseases including glaucoma, retinitis pigmentosa [4], and while there has been an emphasis on changes to the retinal vasculature in diabetes, recent findings reveal neurodegeneration of the retina in DR.

Retinal function changes before the onset of clinically detectable vascular lesions associated with DR. For example, the amplitude and latency of oscillatory potentials in the electroretinogram is impaired in both humans and animals with diabetes [5], and the reasons for these changes have been obscure, but recent cell biological studies suggest possible explanations. For example, the number of apoptotic nuclei in the retina is elevated after only 4 weeks of streptozotocin-induced diabetes in the rat [6], the rate of apoptosis is essentially constant over 12 months of diabetes, and the amount of apoptosis was reduced by the treatment with subcutaneous insulin implants. The majority of TUNEL-positive cells did not colocalize with an endothelial cell-specific marker. This finding suggests that most of the cells undergoing apoptosis were not vascular and more likely to be either neurons or glial cells. Consistent with this finding, the number of retinal ganglion cells (RGCs) and the thickness of the inner retina were reduced after 8 months of diabetes, and the total number of RGC bodies was reduced by 10% compared to age-matched controls, suggesting a reduction in the total number of neurons. There was also a 50% reduction in the total number of cells exhibiting immunoreactivity for NeuN, a cell-specific marker expressed in the nuclei of neurons, in rats with 4 months' diabetes, and a 20% reduction in the NeuN-positive RGCs. The loss of RGCs is also reflected by a reduction in the number of axons in the optic nerve. A similar increase in apoptosis was also observed in postmortem retinas from patients with diabetes. At scanning laser polarimetry, the thickness of the nerve fiber layer in the superior polar quadrant of the retina was significantly reduced in the group of patients with diabetes compared to the control group [7]. Thus, these results clearly indicate that diabetes induces a chronic neurodegeneration in the retina, and although the relationship to specific visual defects remains uncertain, it is likely they contribute directly to altered function in some way.

Normal Mechanisms for Maintenance of Postmitotic Neurons in the Retina

Intact Blood-Retinal Barrier
The BRB is required to maintain precise neuronal and glial cell exchange of neurotransmitters and metabolites in the neural retina. The BRB is formed by the retinal pigmented epithelium (RPE) and the blood vessels of the retina, which form the outer
and inner barriers, respectively. The RPE controls the flow of solutes and fluid from the choroidal vasculature into the outer retina and the retinal vasculature directly controls flux into the inner retina. This strict control of solutes and fluids that cross the BRB is achieved through well-developed intercellular tight junctions. Tight junctions control flux across both endothelial and epithelial cells creating defined environments such as the neural retina. They provide a barrier function restricting the paracellular flux of molecules and a fence function maintaining apical and basolateral membrane composition within a cell. Over 40 proteins are associated with tight junctions, including occludin, claudins and zonula occludens. Occludin and the family of claudins, both of which are transmembrane proteins, are responsible for the direct cell-to-cell attachment in the establishment of the tight junction barrier. Occludin is expressed predominantly on epithelial and endothelial cells and its content correlates with the degree of barrier properties [8]. As many as 23 isoforms of claudins have been described and shown to be implicated in the control of ion and small molecule movement through the tight junction barrier [9]. The wide distribution of different claudins in various tissues with a wide range of different barrier properties suggests that these proteins control much of the tissue barriers characteristics. Zonula occludens are intracellular proteins that associate with the cytoplasmic surface of the tight junctions and organize the tight junction complex. Thus, the BRB contributes to the normal retinal homeostasis and tight junction proteins are important dynamic regulators of the BRB.

Regulated Metabolite Delivery
Glucose is an essential metabolic substrate of all mammalian cells and neural tissue including the retina depends entirely on glucose under normal physiological conditions. The glucose supply to the retina derives entirely from the circulation, and both outer and inner BRBs regulate the passive diffusion of glucose and other blood-borne nutrients into the interstitial space surrounding neural cells. Glucose entry is mediated by specific transport process involving members of the sodium-independent glucose transporter (GLUT) family. Five functional isoforms of the GLUT family have been identified. In the retina, GLUT1 is expressed in the retinal capillary endothelial cells and the RPE. In the human inner BRB, a substantial portion of total cellular GLUT1 resides in cytosolic stores. These transporters may rapidly translocate to the plasma membrane where they mediate glucose entry into endothelial cells in response to external stimulation. GLUT1 is also expressed in RGCs, photoreceptors and Müller cells. The finding of abundant expression of GLUT1 in Müller cells is in line with evidence of glucose transport and metabolism in isolated cell preparations. Glial cells support neuron function in numerous ways, so this result illustrates one way in which Müller cells serves as a nutrient-supporting cell of the retina [10].

In addition to controlling retinal glucose delivery, the BRB also regulates access of amino acids to the retina that are used as neurotransmitters and protein precursors. The BRB prevents excessive retinal access by circulating antibodies and immune cells.
**Excitatory Neurotransmitters**

Amino acids or their metabolites have a role as neurotransmitters and are involved in normal synaptic functions under physiologic conditions. Glutamate works as an excitatory neurotransmitter in the central nervous system, including the retina [11], in which photoreceptors, bipolar cells and ganglion cells release glutamate. The synaptic function by glutamate is mediated through various postsynaptic receptors, including the N-methyl-D-aspartate (NMDA) receptor, and terminated by its re-uptake from the synaptic cleft into surrounding cells by the excitatory amino acid transporter (EAAT). Meanwhile, if excessive amounts of glutamate are released from injured nerve cells or if glutamate re-uptake by EAAT is not sufficient, neuronal death can result in a process known as glutamate-mediated excitotoxicity. The NMDA receptors are associated with this excitotoxicity and calcium entry into the cells, which finally causes apoptosis of the cells, and the re-uptake system by EAAT is essential to keep the extracellular glutamate concentration, to control the specificity of glutamate effects and to limit the potentially damaging effects of lingering glutamate, including the neurodegeneration [11]. Thus, in retina, glutamate contributes to both the physiological synaptic function and excitotoxicity induced in several ocular neurodegenerative disease including glaucoma and DR.

**Neurotrophins**

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is protein identified initially as an endothelial cell mitogen and vascular permeability factor. In the retina, VEGF is produced by the RPE, pericytes, RGCs, Müller cells and endothelial cells in normal conditions and its expression is regulated in response to hypoxic conditions. It is well known that VEGF is a mediator of angiogenesis, BRB breakdown, inflammation, and leukocyte adhesion. In addition, VEGF affects neuronal growth, differentiation and survival. In vitro, VEGF stimulates axonal outgrowth, improves the survival of ganglion neurons, and can rescue hippocampal neurons from apoptosis induced by serum withdrawal. In vivo, VEGF coordinates migration of motor neuron soma and local delivery of VEGF prolongs motor neuron survival. Conversely, low VEGF levels have been linked to the neuronal degeneration in both animal models and human disease [12]. These results suggest an important role for VEGF in neuronal development and maintenance within the central nervous system. The receptors for VEGF are present in normal retinal neuronal cells, indicating a possible functional role for VEGF in the neural retina [13]. Gene expression studies in the brain and retina also suggest that VEGF is upregulated by hypoxia preconditioning, a brief ischemic episode that protects neurons, against subsequent prolonged ischemia-reperfusion-related damage. Thus, VEGF might provide neuroprotection in the retina, particularly during ischemia or other metabolic stresses. In fact, glucosamine stimulates VEGF expression in retinal neurons, possibly...
via RNA-dependent protein kinase-like endoplasmic-reticulum associated kinase (PERK) [14], so ischemia is not the only stimulus for VEGF expression.

Erythropoietin
Erythropoietin (EPO) is a member of the cytokine superfamily, and has been known best as a hematopoietic cytokine produced by fetal liver and adult kidney. Recently, it has been shown that EPO and EPO receptor are also expressed in the human central nervous system including retina. In a model of retinal ischemia, EPO prevented apoptotic cell degeneration, and improved functional recovery. Likewise, light-induced photoreceptor degeneration was reduced by systemic pre- or post-treatment with recombinant EPO [15]. EPO-mediated neurotrophic effects have been attributed to pleiotropic mechanisms, including interactions with Jak2, Akt/PKB, ERK1/2, and Bcl-XL, increased calcium influx, reduction of nitric oxide (NO) production, restoration of glutathione peroxidase activity, and IL-6 suppression.

Insulin and Insulin-Like Growth Factors
Insulin is a potent anabolic hormone synthesized in the pancreatic beta cells that stimulates the uptake and storage of carbohydrates, fatty acids, and amino acids into glycogen, fat, and protein, respectively. The insulin supply to retina is thought to depend on the delivery from the circulation, although several studies have suggested the possibility of insulin production in other extrapancreatic tissues including retinas [16]. However, our studies have not found detectable levels of pre-proinsulin mRNA in normal rat retina [unpubl. data]. In normal conditions, insulin-like growth factor-1 (IGF1) and IGF2, which are 60% homologous at the amino acid level and have significant homology to insulin, are expressed in several tissues including the retina. IGF1 can mimic the metabolic effect of insulin. The activity of all three ligands is mediated through the insulin receptor (IR) and insulin-like growth factor receptor-1 (IGF1R), both belonging to the transmembrane tyrosine kinase receptor family. Ligand binding and subsequent autophosphorylation of IR and/or IGF1R leads to activation of several intracellular signaling pathways including phosphoinositide-3 kinase (PI3-K) and/or mitogen-activated protein kinase (MAPK).

We demonstrated Akt-dependent cell survival in cultured retinal neurons in response to insulin and IGF1 [17] in a PI3-K/Akt-dependent manner and reduced apoptosis induced by serum deprivation. Physiological concentration of insulin increases IR beta autophosphorylation, Akt<sup>Ser473</sup> phosphorylation in the retinal nuclear layers and Akt-1 kinase activity in ex vivo cultures [18]. Inhibition of p70 ribosomal protein S6 kinase (p70S6K) activity, a protein synthesis regulator, using a dominant negative mutant, prevented insulin-induced cell survival, whereas overexpression of wild-type p70S6K enhanced the effect of insulin on survival. Meanwhile, IGF1 and its analog inhibited the neuroretinal cell death in vitro, and intraocular injection of IGF1 protected retinal ganglion cells from axotomized-induced secondary death via PI3-K dependent Akt phosphorylation and by inhibition of caspase-3
Thus, insulin and IGF1 have a neuroprotective effects via the IR/PI3-K pathway but the physiologic roles of these ligands remains incompletely defined.

**Pigment Epithelium-Derived Factor**

Pigment epithelium-derived factor (PEDF) is a 50-kDa glycoprotein that belongs to the superfamily of serine protease inhibitors. PEDF was first identified in conditioned medium from fetal human RPE cell cultures with neuronal differentiating activity [20], and is widely synthesized in neural and non-neural tissues. In the retina, its expression is detected in RPE, photoreceptor and ganglion cells. PEDF can promote activation of nuclear factor κB (NF-κB), a transcription factor that in turn induces the expression of antiapoptotic genes and/or neurotrophic factors in cerebellar granule cells. PEDF triggers NF-κB activation through increased phosphorylation and decreased protein levels of the inhibitor of NF-κB (IκB) as part of its mechanism to protect immature cerebellar granule cells against apoptosis. PEDF also protects rat retinal neurons from hydrogen peroxide-induced cell death in culture. PEDF delays the death of photoreceptor cells in models of retinitis pigmentosa, retinal degeneration (rd/rd), and retinal degeneration slow (rds/rds) mice [21]. PEDF protects rat photoreceptors cells from light damage [22]. PEDF also protects retinal neurons from transient ischemic reperfusion [23]. Thus, PEDF has neuroprotective roles in both acute and chronic forms of neurodegeneration.

**Brain-Derived Neurotrophic Factor**

Brain-derived neurotrophic factor (BDNF) is widely expressed throughout the central and peripheral nervous systems. In the retina, BDNF is expressed in RGCs, amacrine and Müller cells [24]. BDNF binds with high affinity to the TrkB receptor, which is expressed in RGCs, amacrine cells and the RPE, and in cones but not rods. TrkB receptor signaling by BDNF occurs through both PI3-K and MEK/ERK pathways, and suppresses the activation of caspase-3. BDNF provides autocrine and paracrine trophic support to RGCs. Upregulation of Trk receptor gene expression appears to be a normal response of RGCs to axotomy and BDNF enhances the survival of RGCs after optic nerve injury [25]. Transgenic expression of BDNF prolongs the survival of RGCs after experimental axotomy [25], and axon-mediated gene transfer of a Trk receptor into RGCs via retrograde transport from axonal terminal arbors delays axotomy-induced apoptosis [26]. TrkB gene transfer into RGCs together with administration of exogenous BDNF also increases RGCs survival. Thus, BDNF is involved in normal maintenance of the retina.

**Alterations in Normal Cellular Maintenance Pathways in Diabetic Retinopathy**

**Dysregulated Blood-Retinal Barrier**

Breakdown of the BRB occurs early in the course of diabetes, before retinopathy is observed clinically. Several permeabilizing factors such as histamine, IL-1, tumor
necrosis factor-α and VEGF have been implicated as causes of breakdown of the BRB [27]. The reduced expression and anatomic distribution of occludin in experimental diabetes occur in a timeframe concurrent with early neurodegenerative changes. A similar decrease in occludin and ZO-1 tight junction protein content was also noted upon VEGF treatment of cultured brain microvessel endothelial cells, coincident with increase permeability of the cellular monolayer. Redistribution of occludin from the plasma membrane to the endothelial cell cytoplasm, a phenomenon that is accompanied by increased occludin phosphorylation, is observed with both diabetes and with VEGF treatment [28]. Intravitreal injection of VEGF in rats increased phosphorylation of ZO-1 and occludin. All of these modifications may be critical for the breakdown of the BRB in diabetes, and VEGF likely facilitates this breakdown.

*Altered Glucose Metabolism*

Glucose accumulates to a supranormal intracellular level in diabetes as a result of an increased uptake, a decreased efflux, or metabolism of intracellular glucose. Retinal glucose also increases in diabetes in the extracellular space as a result of increased permeability of the BRB [29]. GLUT1 mRNA abundance has been reported to be increased in the retina of diabetic rats. In streptozotocin-induced diabetic rats, decreased total retinal GLUT1 and retinal microvascular GLUT1 were reported [30]. On the other hand, a localized GLUT1 increase on endothelial cells of the inner BRB was demonstrated in postmortem retina from patients with diabetes [31]. These results may suggest that the upregulation of particular inner BRB GLUT1 occurs in the early stages of DR, and that the resultant increase in inner BRB glucose transport could play a role in the progression of the DR.

*Altered Glutamate Metabolism*

As mentioned above, glutamate-mediated excitotoxicity has been shown to play an important role in several ocular diseases including DR. Elevated glutamate concentration was detected in human vitreous samples in patients with PDR and in the retina of streptozotocin-induced diabetic rats. At the same time, reduced glutamate oxidation and glutamine synthesis is due to transaminase and glutamine synthetase dysfunction in streptozotocin-induced diabetic rats [32]. Additionally, increased immunoreactivity of NMDA receptor subunit-1 was observed in both outer and inner plexiform layers in donor eyes from subjects with diabetes [33], and in ganglion, amacrine and bipolar cells in streptozotocin-induced diabetic rats. Moreover, significantly decreased activity of the Müller cell glutamate transporter, as analyzed by the patch-clamp technique, was detected in streptozotocin-induced diabetic rats. Taken together, these data suggest that the abnormal glutamate metabolism induced by diabetes may contribute to the neurodegeneration in DR.
Disturbances of Neurotrophin Function

As detailed in the preceding paragraphs, peptide growth factors are expressed throughout the retina as part of normal homeostasis. So what are the implications of increased expression of these factors in DR? We hypothesize [34] that they increase as an adaptive response to the metabolic alterations of diabetes in an attempt to maintain retinal viability, but a maladaptive state ensues when the diabetes persists, resulting in excessive growth factor production. This overproduction may then result in the clinical features of macular edema and neovascularization. Moreover, merely finding an increased ligand concentration does not necessarily imply increased ligand receptor activity because soluble receptors can also be overexpressed or the receptor activity may be downregulated [35]. Thus, neurotrophins serve multiple roles and their net effects may depend on the context of other cellular events.

VEGF

Clinical studies have revealed that intravitreal VEGF concentration increases in subjects as they progress from nonproliferative DR to active proliferative diabetic retinopathy (PDR) [36]. In diabetic rats, the levels of VEGF and its receptor VEGFR2 in the retina are increased [13]. The increased VEGF expression is strongly correlated with increased vascular permeability following the breakdown of BRB and the neovascularization.

Erythropoietin

An elevated EPO concentration was detected in human vitreous samples in patients with PDR and diabetic macular edema [37]. Additionally, the expression of the EPO receptor was increased in streptozotocin-induced diabetic rat retinas, whereas the expression of the EPO protein was unchanged. These results suggest that the EPO and EPO receptor which are induced in hypoxic conditions are also induced during the early stages of diabetes.

Insulin/Insulin-Like Growth Factors

The intravitreal insulin concentration was unchanged in patients with type 2 diabetes who were not receiving insulin treatment compared to controls [38]. On the other hand, a reduced intravitreal insulin concentration was detected in streptozotocin-induced diabetic rats. These results may suggest that type 2 diabetic patients may have normal vitreous insulin concentration, whereas type 1 patients may have reduced vitreous insulin, but the vitreous insulin levels were not corrected for plasma insulin concentrations in the human study. Studies of IGF1 concentration in the serum in diabetic subjects are still controversial. Several studies suggest that patients with PDR have elevated levels of IGF in serum [39]. On the other hand, patients with type 1 diabetes usually demonstrate reduced levels of serum IGF1 because of decreased hepatic production [40]. The existence of insulin-like growth factor-binding proteins makes it difficult to measure the correct concentration of free IGFs and could explain these discrepancies. In the vitreous, an increased concentration of IGF1 was detected in patients with PDR [41].
The abundance of IGF1 mRNA was significantly higher in retina from diabetic rats compared to age-matched controls. On the other hand, reduced retinal levels of IGF1 protein were detected in diabetic animals. These differences may be due to differences in the duration of diabetes as well as the type of diabetes in the animals.

Pigment Epithelium-Derived Factor
Intravitreal PEDF concentration was decreased in patients with PDR and in experimental diabetic rats [42], whereas it was significantly higher in experimental diabetic rat retinal tissue. On the other hand, inhibition of VEGF-induced and advanced glycation end products (AGE)-elicited retinal leukostasis by PEDF was reported. This result suggests that controlling PEDF levels might be a valuable approach to managing some neurological diseases including DR.

Brain-Derived Neurotrophic Factor
In diabetic rat retina, BDNF protein expression was decreased whereas TrkB receptor expression was not altered [24]. BDNF immunoreactivity was decreased in diabetic rat retinas as were TH-1 and Thy-1 protein levels, markers of dopaminergic amacrine cells and RGC cells, respectively. Intravitreal BDNF injection restored TH-1 protein expression and the TH-1-positive cell density. These results suggest the involvement of a reduced BDNF expression in the development of DR and specifically its neurodegenerative aspect.

Neuroprotective Strategies for Diabetic Retinopathy Treatment

Control of Retinal Glucose Metabolism
Glucose is the primary energy substrate in the retina so appropriate regulation of its metabolism is central to the viability of retinal cells. Impaired glucose homeostasis is a major systemic consequence of the altered insulin action in diabetes. Retinal glucose concentrations are increased in diabetic rats [43] but glycolytic flux is reduced in retinas of diabetic animals compared to controls [44]. Moreover, retinal glucose metabolism is linked closely with that of glutamate and branched chain amino acids [45] and thus very complex. However, at this point there are insufficient data to provide a thorough understanding of the defects in retinal glucose metabolism in either type 1 or type 2 diabetes.

Actions of Systemically Administered Insulin
Diabetic retinopathy in experimental rats involves loss of constitutive retinal insulin receptor signaling that is restored by systemic and intravitreal insulin [18]. Insulin provides trophic support for retinal neurons in culture [17] and systemic insulin treatment reduces retinal apoptosis in diabetic rats [6]. Intrathecal delivery of low-dose insulin, insufficient to reduce hyperglycemia, improves peripheral nerve conduction
velocity in diabetic rats and sequestering intrathecal insulin in nondiabetic rats using an anti-insulin antibody slows conduction velocity and axonal atrophy resembling changes in diabetes. Thus, insulin appears to have direct neuroprotective effects for the central nervous system independent of those related to glucose metabolism.

The DCCT showed a lower risk of retinopathy progression in type 1 diabetic patients who received intensive diabetes therapy (3–4 insulin injections daily or a pump) than those receiving conventional treatment (1 or 2 daily injections) [46]. This reduced risk was achieved by administering insulin more frequently to the intensive treatment group and this lower risk persisted in the intensive treatment group patients for the same HbA1c levels as those in the conventional treatment group. Moreover, the risk of progression was lower in patients in whom insulin treatment was initiated at an earlier stage of disease, when no clinically evident retinopathy was present, compared to those in whom intensive treatment began after the onset of mild-to-moderate nonproliferative retinopathy. Hence, the DCCT might be viewed as an insulin dose-response trial [47]. Further clinical evidence for a role of insulin derives from the observation that systemic insulin resistance is a risk factor for retinopathy in patients with type 1 diabetes in the DCCT [48], the EURODIAB study [49], and the Pittsburgh Epidemiology of Diabetes Complications study [50]. In type 2 diabetes, insulin deficiency is also an independent risk factor for the presence of retinopathy [51]. These data suggest that the retina is an insulin sensitive tissue and retinal insulin signaling provides neurotrophic support.

**Sigma Receptor 1 and Its Ligand, (+)-Pentazocine**

Sustained (+)-pentazocine treatment in Ins2Akita/+ diabetic mice restored the disruptions of retinal architectures induced by DR, like decrease of the inner plexiform layer and inner nuclear layer thickness, reduction of ganglion cells, and loss of the uniform organization of radial Müller fibers [52]. This result may suggest the potential use of (+)-pentazocine, which is a ligand of sigma receptor 1, for the treatment of neurodegenerative disease including DR.

**Future Directions**

Phase 1 trials of ciliary neurotrophic factor (CNTF) for treatment of retinitis pigmentosa highlight the promising potential of neurorophins to treat retinal neurodegeneration [53]. Of all the major retinal diseases, DR is the only one for which large-scale clinical trials have proven the ability to reduce disease incidence and progression [46]. Neurodegeneration in DR starts before the onset of clinically evident disease [6] and this early phase of disease offers the greatest window of opportunity for treatment before significant vision loss has occurred. Any therapeutic neurotrophin must act to restore retinal physiology without serious side effects, and it is plausible that neurotrophins may have different therapeutic potentials at different stages of DR. It is
unlikely that a single neurotrophic agent would correct the myriad of neurovascular degenerative changes in DR. Further, neurotrophin changes in DR such as increased VEGF medicated vascular permeability may represent adaptive responses that have become maladaptive over time. A better understanding of the role of neurotrophins in retinal physiology would be key to devising therapeutic interventions in disease.

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References


Neuroprotection for Diabetic Retinopathy


VEGF Inhibitors and Vitrectomy for Diabetic Vitreoretinopathy

Carlos E. Cury, Jr³ · Eduardo B. Rodrigues[a] · Carsten H. Meyer[b] · Michel E. Farah[a]

[a]Vision Institute, Department of Ophthalmology, Federal University of Sao Paulo, Sao Paulo, Brazil; [b]Department of Ophthalmology, University of Bonn, Bonn, Germany

Abstract

Diabetic retinopathy (DR) is one of the leading causes of blindness worldwide. Breakdown of the blood-retinal barrier in DR promotes accumulation of a high concentration of pre-retinal serum-derived chemoattractants, thereby stimulating cellular migration on the attached posterior hyaloid. This review assesses the role of intravitreal application of vascular endothelial growth factor (VEGF) inhibitors in combination with surgical removal of the vitreous. Vitrectomy with removal of the posterior hyaloids wash out the pocket of preretinal growth factors and enhance the diffusion of macromolecules, including VEGF, insulin-like growth factor 1 or histamines, from the retinal into the vitreous cavity for further absorption through the anterior segment outflow pathways. The release of tractional forces induced by the vitreomacular traction or epiretinal membranes demonstrated a strong correlation to the reduction of retinal thickness in DME. Three techniques are described to remove the pre-retinal thickened and adherent vitreous: (1) delamination, (2) segmentation, and (3) en block dissection. A better visualization of remaining cortex vitreous or adjacent epiretinal membrane and safer removal may be achieved by a better intraoperative visualization using a variety of vital dyes to satin retinal tissue (chromovitrectomy). Anti-VEGF treatments may represent an alternative adjunctive treatment for DR.

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Diabetic retinopathy (DR) is one of the leading causes of blindness worldwide and the most common microvascular complication of diabetes [1]. During the first two decades of the disease, nearly all patients with type 1 diabetes and over 60% with type 2 diabetes develop retinopathy. In the Wisconsin Epidemiologic Study of Diabetic Retinopathy, also named WESDR, 3.6% of younger-onset patients and 1.6% of older-onset patients were blind [1]. Duration of diabetes and severity of hyperglycemia are the major risk factors for DR, while others include age, type of diabetes, clotting factors, and renal disease [2]. Timely application and reapplication of laser photocoagulation is the mainstay of treatment to reduce visual loss and to avoid the need for vitrectomy in patients with more advanced complications of diabetic retinopathy [3]. However, despite preventive
regimens and timely treatment, substantial numbers of eyes will develop complications of proliferative retinopathy and may become candidates for vitrectomy [4].

Due to the limitations of current treatment approaches, new pharmacological therapies have been developed. The novel drugs target specific biochemical pathways that cause DR through involvement of protein kinase C (PKC) activation, oxidative stress, the angiogenesis pathway, and the glycation and sorbitol pathway. These treatments aim to prevent diabetes-induced damage to the retinal microvasculature [5]. In this chapter the role of intravitreal application of vascular endothelial growth factor (VEGF) inhibitors in combination with surgical removal of the vitreous will be discussed.

Role of the Vitreous and Various Factors Including VEGF in Diabetic Vitreoretinopathy

In healthy eyes, ultrastructural components of the vitreous can be observed histologically or histochemically, and basically they are mainly collagen type II, hyaluronan acid and hyalocytes [6]. In diabetic eyes, consecutive studies have demonstrated elevated levels of various cytokines such as VEGF, insulin-like growth factor-1 and histamines [7]. Pars plana vitrectomy with consecutive removal of the posterior hyaloids may resolve the diabetic macular edema (DME) and improve the central vision of patients who previously failed to respond to conventional laser photocoagulation treatment.

Four theories may support this hypothesis [7]:

Mechanical Release Theory. Breakdown of the BRB in DR promotes accumulation of a high concentration of preretinal serum-derived chemoattractants, thereby stimulating cellular migration on the attached posterior hyaloid. In addition, molecular changes in the vitreous induced by hyperglycemia may thicken and enhance its adherence to the retinal surface. Sebag et al. [9] suggested that the abnormal cross-linking might affect the collagen architecture structure in diabetic vitreous. The contraction of retinal cells, preretinal elements and the vitreous may induce tangential and anteroposterior traction and exacerbation of DME with vitreomacular traction (VMT) [8–10]. Release of tractional forces induced by the vitreous or epiretinal membranes demonstrated a strong correlation to the reduction of retinal thickness in DME. Moreover, several retrospective clinical studies have shown that vitrectomy and removal of the posterior hyaloid may lead to visual improvement of 2 lines in 38–92% of the eyes. Recently, Tachi and Ogino [11] observed the resolution of DME eyes within 3 months after vitrectomy without inner limiting membrane (ILM) peeling and questioned whether there is an additional beneficial effect of ILM removal.

Chemical Diffusion Theory: In eyes with diabetic retinopathy there may be an abnormal concentration of growth factors under the abnormally attached premacular posterior hyaloid thereby exacerbating DME. Vitrectomy with removal of the posterior hyaloids and vitreous gel may release and wash out the pocket of preretinal growth factors and enhance the diffusion of macromolecules, including VEGF, insulin-like
growth factor 1 or histamines, from the retinal into the vitreous cavity for further absorption through the anterior segment outflow pathways. In 1992, Lewis et al. [12] described a positive effect on visual acuity of pars plana vitrectomy on diabetic patients who had a thickened and taut posterior hyaloid traction on the macula. In the following years, numerous retrospective studies have shown the advantage of vitrectomy and removal of the posterior hyaloid on morphologic and functional results [13, 14].

Decrease or Wash-Out of Pro-Angiogenic Growth Factors – VEGF. Previous reports by Funatsu’s group have associated the aqueous VEGF concentration or determined its vitreous concentration in patients with DR and DME. A clear relationship has been established between structural and molecular parameters demonstrating that there are differences in the concentration of cytokines between DME and normal eyes. VEGF, a vascular endothelial cell mitogen and potent permeability factor, is produced by glial cells, retinal pigment epithelial cells, and vascular endothelial cells and is normally present in the retina and vitreous in low levels. Retinal hypoxia upregulates VEGF production, which results in abnormal angiogenesis, may increase in vascular permeability. Chronic hyperglycemia of uncontrolled diabetes leads to increased cellular levels of diaclylglycerol, which in turn increases the synthesis of VEGF and also contributes to the microvascular abnormalities in DR. Inhibition of either VEGF moderates the microvascular complications seen in experimental animal models. Recent work also disclosed elevated levels of VEGF in ocular fluids of patients with proliferative diabetic retinopathy (PDR) [15]. These studies also point out the growth of new vessels from the retina or optic nerve to occur as a result of VEGF release into the vitreous cavity as a response to ischemia. Furthermore, injection of VEGF into normal primate eyes induces the same pathologic processes seen in diabetic retinopathy, including microaneurysm formation and increased vascular permeability [16]. In addition, experiments in animals have suggested a central role for the 165 isoform of VEGF specifically in the pathogenesis of DME, and increased retinal VEGF164 (the rodent equivalent to primate VEGF165) levels in this model coincide temporally with breakdown of the BRB [17]. Clinical studies demonstrate that intravitreal VEGF concentration increased as subjects progressed from early diabetic retinopathy to active PDR [16]. Gandorfer et al. [13] reported on the resolution of DME after vitrectomy in eyes without any evidence of VMT. The characterization of molecular and cellular events causing retinal microvascular abnormalities in DR has provided new targets for pharmacologic manipulation.

Oxygenation Hypothesis. Experimental data suggest that both scatter laser treatment and vitrectomy have a positive effect on the improvement of retinal oxygenation. Stefansson et al. [18], performed pars plana vitrectomy on one eye in cats and created a branch retinal vein occlusion in both eyes while the retinal oxygen tension was measured simultaneously in both. In the nonvitrectomized eye the branch retinal vein occlusion led to severe hypoxia of the retina. In the eye where vitrectomy had been performed there was insignificant change of the retinal oxygen tension with the vein occlusion. The beneficial effect of vitrectomy on DME can be further understood through improved oxygenation of the retina. Following vitrectomy (or a posterior
vitreous detachment) the ischemic retina receives oxygen from the vitreous cavity and the oxygenation of the tissue is increased. The increased oxygen level leads to reduced VEGF production and decreased vascular permeability as well as arteriolar vasoconstriction and reduced blood flow, with decreased hydrostatic pressure in the capillaries and venules and reduced edema formation. Vitrectomy alleviates hypoxia in ischemic areas of the retina which may reduce VEGF production in these areas and decreases new vessel formation [18].

Anti-VEGF Agents

A key molecule being targeted in current clinical trials is VEGF. Anti-VEGF treatments may represent as an alternative adjunctive treatment for DR and three presently available anti-VEGF agents are pegaptanib, bevacizumab and ranibizumab [19, 20].
**Fig. 2.** Color retinography of a 32-year-old type 1 DM patient. 

- **a, b** Right and left eye demonstrating a TRD affecting the macula. 
- **c, d** 30th postoperative day view of right and left eye submitted to PPV plus membrane dissection and avastin 1.25 mg.

**Fig. 3a–c.** Color retinography and angiography of a only eye patient with very severe PDR. 

- **d, e** Color retinography and late phase angiography 15 days after avastin injection, demonstrating involution of the fibrovascular complex. 
- **f** 15th day post-PPV plus membrane dissection color retinography.
Pegaptanib

An inhibition of VEGF165 can be accomplished with an aptamer, a new therapeutic class of highly selective nonbiologic agents. Pegaptanib sodium is the first FDA-approved ophthalmologic anti-VEGF agent for the treatment of choroidal neovascularization (CNV) from age-related macular degeneration (AMD). Animal model studies have shown that intravitreal injection of pegaptanib can decrease the breakdown of the BRB characteristic of diabetes and can even reverse this damage to some degree. A recent phase II study reported that intravitreal pegaptanib has promising results for DME when given every 6 weeks for 30 weeks. The results showed better visual acuity at week 36 with 0.3 mg injection (20/50) as compared with sham injection (20/63) \( (p = 0.04) \); a mean central retinal thickness decrease of 68 μm with 0.3 mg, versus an increase of 4 μm with sham \( (p = 0.02) \); and reduction of subjects requiring photocoagulation in each pegaptanib arm \( (0.3, 1, 3 \text{ mg}) \) [21]. Prospective clinical investigation should disclose the role of pegaptanib for the management of DME. Retrospective analysis of these data demonstrated some efficacy on retinal neovascularization as well. Phase 3 trials of pegaptanib for DME are currently being conducted.

Bevacizumab

Bevacizumab is a full-length recombinant humanized antibody active against all isoforms of VEGF-A. The US FDA approved bevacizumab in February 2004 for treatment for first-line metastatic colorectal cancer [22]. Case reports and small observational series show the efficacy of off-label intravitreal bevacizumab to treat exudative AMD [23], macular edema from nonischemic central retinal vein occlusion [24], iris neovascularization [25] and pseudophakic cystoid macular edema [26]. Haritoglou et al. [27] disclosed a prospective consecutive noncomparative case series of 51 patients treated with bevacizumab for diffuse refractory DME followed for up to 12 weeks. Their results showed a significant reduction in mean retinal thickness, from 425 μm at 2 weeks to 377 μm at 12 weeks \( (p = 0.001) \), although no significant changes in ETDRS letters were noticed throughout follow-up. In a second retrospective multi-center study named PACORES (Pan American Collaborative Retina Study Group), the clinical records of 88 patients with DME treated for 6 months with at least one intravitreal injection of 1.25 or 2.5 mg of bevacizumab were presented. The study found a statistically significant mean improvement in Logmar vision \( (p < 0.0001) \), whereas mean central macular thickness by OCT decreased over 100 μm at the end of follow-up [28]. In contrast to those results, Yanyali et al. [29] also recently showed that intravitreal bevacizumab may not promote improvement of DME or visual acuity in the short-term in 11 vitrectomized eyes. The authors attribute such findings to rapid clearance of intravitreal bevacizumab from the vitreous cavity and thus insufficient sustained therapeutic levels or individual systemic factors that may affect DME. DRCR.net has completed enrollment on a phase
2, prospective, randomized, multicenter clinical trial to determine the safety and possible benefits of this agent. Plans for a phase 3 trial of two doses of an intravitreal anti-VEGF agent versus modified ETDRS grid laser photocoagulation for DME are under discussion.

**Ranibizumab**

Ranibizumab is a fragment of a recombinant humanized IgG1 monoclonal antibody that inhibits all A isoforms of the human VEGF-A. Intravitreal ranibizumab is FDA approved for the treatment of exudative AMD [30]. One pilot study including 10 patients with DME, 5 receiving ranibizumab 0.3 mg and 5 receiving ranibizumab 0.5 mg, disclosed good systemic and ocular drug tolerability. At month 3, 4 of 10 patients gained >15 letters, and 8 of 10 gained >1 letter. At month 3, the mean decrease in retinal thickness of the center point of the central subfield was 45.3 ± 196.3 μm for the low-dose group and 197.8 ± 85.9 μm for the high-dose group [31]. Randomized controlled double masked trials are underway to test whether intraocular injections of ranibizumab provide long-term benefit to patients with DME. DRCR.net is planning two phase 3, prospective, randomized, multicenter trials comparing patients. In the first trial, patients with DME and no PDR will be randomized to: (1) modified ETDRS grid laser photocoagulation; (2) photocoagulation before ranibizumab; (3) photocoagulation plus IVTA, or (4) ranibizumab before photocoagulation. In the second trial, patients with DME and PDR will be randomized to: (1) modified ETDRS grid laser photocoagulation plus scatter photocoagulation; (2) modified ETDRS grid laser photocoagulation plus scatter photocoagulation plus ranibizumab, or (3) modified ETDRS grid photocoagulation plus intravitreal triamcinolone injection (IVTA).

**Corticosteroids and Diabetic Vitreoretinopathy**

Intravitreal injection of triamcinolone acetonide (TA) may decrease DME resulting from various causes, including diabetic retinopathy 1 week to 6 months after TA injection through inhibition of VEGF cytokines. Recent prospective, randomized clinical trials have demonstrated generally favorable outcomes, the Diabetic Retinopathy Clinical Research network (DRCR.net) has completed enrollment on a three-year, randomized, prospective, multicenter clinical trial comparing two doses (1 mg and 4 mg) of preservative-free IVTA with modified early treatment diabetic retinopathy study (ETDRS) photocoagulation for DME. Long-term efficiency and safety reports of repeated intravitreal injections remain uncertain, but encouraging reports promote the development of long-term sustained release devices for corticosteroid therapy. The most important complication of IVTA is increased intraocular pressure resulting in secondary open-angle glaucoma, which sometimes may be severe and intractable.
Elevation of IOP up to 24 mm Hg may occur in about 40% of patients, usually within about 3 months. The second most important complication of IVTA is cataract formation, which may become visually significant in about half of eyes within 1 year. Other reported complications of IVTA include endophthalmitis, pseudoendophthalmitis, retinal detachment, lens trauma, and vitreous hemorrhage.

Dexamethasone is theoretically a more potent corticosteroid than TA, and intravitreal injections of dexamethasone have been shown to produce high intravitreal drug levels without toxic effects. Unfortunately, the short intraocular half-life of dexamethasone after intravitreal injection (approximately 3 h) makes this approach less useful for clinical therapy. A phase 6-month study randomized 315 patients with persistent DME with visual acuity of 20/40 to 20/200 in the study eye to observation or a single treatment with a dexamethasone implant called DDS, 350 or 700 μg. At day 90 (primary end point), an improvement in VA of 10 letters or more was achieved by a greater proportion of patients treated with dexamethasone-DDS. Results were similar in patients with diabetic retinopathy, vein occlusion, or uveitis or Irvine-Gass syndrome. During 3 months of observation, 11% of treated patients and 2% of observed patients had intraocular pressure increases of 10 mm Hg or higher. The study reported that Dexamethasone-DDS, 700 μg, may have potential as a treatment for persistent macular edema.

**PPV for DR: Surgical Techniques**

The diabetic retinopathy vitrectomy study (DRVS) demonstrated the efficacy of vitrectomy for severe proliferative diabetic retinopathy with non-clearing hemorrhage and progression despite laser treatment [20, 32]. At the time of the DRVS up to 20% of eyes progressed to no light perception. Today many advances have improved our success rates, including the advent of high-speed vitreous cutters, endolaser, wide-field viewing systems and pharmacological management of fibrin, retinal neovascularization and retinal edema.

The main objectives of vitrectomy are to remove media opacities, for control of active progressive PDR completely relieve all tractional adhesions, and manage recurrent complications from previous vitrectomy [33]. There are now reports in the literature suggesting that vitrectomy surgery may be helpful in eyes with refractory macular edema in eyes not responsive to laser photoacoagulation.

**Techniques for Membrane Dissection: Delamination, Segmentation and en-bloc Removal**

The three techniques to remove the pre-retinal thickened and adherent vitreous are (1) delamination, (2) segmentation, and (3) en block dissection. (1) Delamination
starts with the removal of the partially detached posterior vitreous surface between the vitreous base and the edge of the fibrovascular adhesions. Using bimanual techniques, from anterior to posterior, the edge of the fibrovascular membrane is reflected and then it can be grasped with a forceps and avulsed. (2) Segmentation is a technique used to release retinal traction caused by preretinal fibrovascular proliferation. Anteroposterior traction then is released by circumferentially cutting the posterior vitreous surface around the area of epiretinal proliferation followed by cutting of the posterior vitreous surface between epicenters of fibrovascular adhesion, leaving islands of fibrovascular tissue. (3) During an en bloc technique, the anteroposterior vitreous traction may be used to elevate the edge of the fibrovascular membrane. Initially, the vitrectomy cutter is used to create a tunnel through the formed vitreous from the sclerotomy site to an area of vitreoretinal separation. The remaining posterior vitreous surface is left attached and assists in visualizing epicenters of adhesion between fibrovascular tissue and the retina. Horizontal scissors are used to amputate epicenters of fibrovascular vitreoretinal adhesion. A better visualization of remaining cortex vitreous or adjacent epiretinal membrane may be visualized by intraoperative staining with a variety of vital dyes (Chromovitrectomy). While fluorescein stains the vitreous collagen, trypan blue may assist to visualize cellular structures of epiretinal membrane. The ILM may be stained with indocyanine green or brilliant blue [34]. After the posterior vitreous surface and the entire fibrovascular membrane are freed from the retina, they may be removed using the vitrectomy probe.

**High-Speed Cutters**

Several high-speed vitreous cutters are now available for diabetic vitrectomy and provide the advantage of more controlled vitreous removal and working on the retinal surface to remove complex diabetic membranes using the technique of indentation and delamination for removal of diabetic membranes and the concept of ‘one-step’ diabetic vitrectomy. The high-speed cutters can be used to delaminate membranes from the retinal surface and, in the majority of cases, eliminate the need for forceps, scissors, endodiathermy, healon dissection of membranes and other maneuvers traditionally used to manage these complex patients. Once one becomes experienced with this approach, cautery and most other steps may be usually eliminated.

**Vitreous Base Cleaning**

Scleral depression is performed while viewing through the wide-field lens system to check the retinal periphery for tears. The vitreous cutter should perform a thorough cleaning of the vitreous base to assure that: (1) postoperative retina oxygenation will be efficacious; (2) diffusion of the cytokines from ciliary body to the retina and from
retina to aqueous humor will be allowed, and (3) postoperative hemorrhage will be prevented.

Small-Gauge Vitrectomy

Vitreoretinal surgeons have been developing new and innovative ways to operate with less invasive techniques. Small gauge vitrectomy instrumentation is now available allowing no-stitch surgery. 23- and 25-gauge vitrectomy instruments are now available from Bausch and Lomb, Alcon and others. These systems allow no-stitch transconjunctival insertion of vitreoretinal instrumentation, minimizing trauma to the eye and reducing the number of surgical steps. Each 25-gauge system utilizes a trocar-cannula system placed directly through the displaced conjunctiva as ports for instruments and infusion. Advantages of 25-gauge vitrectomy include a quieter eye with minimal patient discomfort and potentially less postoperative inflammation with more rapid visual recovery.

Pharmacologically-Assisted PPV: Anti-VEGF Agents and Corticosteroids

Diabetic Macular Edema

For DME pharmacologic agents including anti-VEGF or corticosteroids may be used intra- or postoperatively. Before surgery there is currently no rationale for the use of pharmacologic agents in DME. During surgery, the white corticosteroid triamcinolone may be injected for better visualization of the semi-transparent vitreous thereby enabling a complete vitrectomy, and this procedure has been recently called chromovitrectomy. Most patients with DR show a multilayered preretinal vitreoschisis. Therefore, when the vitreous is not stained, one or more layers of vitreous may remain, which may hamper postoperative vision improvement in patients with DME. In addition to chromovitrectomy, at the end of vitrectomy either anti-VEGF or TA may be injected into the vitreous cavity as powerful adjuvants for vitrectomy for DME.

Proliferative Diabetic Retinopathy

There is recent evidence for a benefit of preoperative anti-VEGF agents as an adjuvant to vitrectomy for the management of severe PDR [35]. Bevacizumab can potentially inhibit early neovascularization, which might be one of the causes of early recurrent hemorrhage. Then, the chances of postoperative complications such as rebleeding or fibrinoid syndrome may be decreased. It has also been hypothesized that suppression of intraocular VEGF theoretically could reduce the risk of intraoperative hemorrhage
during membrane dissection facilitating the surgery. With a better visibility the surgeon may be less likely to create an iatrogenic retinal break. However, others recently proposed that even though bevacizumab may promote new vessel regression, it is unlikely to prevent early postoperative bleeding from vessels that have been injured mechanically during surgery. They suggest that the use of anti-VEGF at the end of surgery might delay the repairing process of injured blood vessels and potentially induce more recurrent hemorrhage during the early postoperative period.

New vessel formation and evolution may be affected and controlled by various growth factors and cytokines. Some factors may stimulate active vessel proliferation, while others induce fibrosis. When anti-VEGF is used, the balance among different growth factors may tip toward fibrosis inducing factors, causing contraction of the fibrovascular tissue. The manifested result may be more vitreoretinal traction or frank traction RD or even combined detachment. Arevalo et al. [36] concluded that tractional retinal detachment may occur or progress very shortly following administration of intravitreal bevacizumab in patients with severe PDR. The development or progression of tractional retinal detachment (TRD) in PDR following intravitreal bevacizumab could have happened by rapid neovascular involution with accelerated fibrosis and posterior hyaloidal contraction as a response to decreased levels of VEGF.

Based on that case series and their hypothesis, the PPV should be conducted no more than seven days after the injection of anti-VEGF agents to avoid the fearful complication of TRD.

Conclusions

In DR, the gathering of preretinal serum-derived chemoattractants promotes stimulation of cellular migration on the attached posterior hyaloid. VEGF inhibitors in combination with surgical removal of the vitreous may be a reasonable approach to remove the pocket of pre-retinal growth factors and enhance the diffusion of macromolecules, including VEGF, insulin-like growth factor 1 or histamines from the retina into the vitreous. In addition, the release of tractional forces induced by the vitreomacular traction promotes additional vision improvement and reduction of macular thickness in DME. Sutureless 23–25 gauge instruments allowed the surgeon to be less aggressive to the tissues, reducing the surgical trauma and consequently less inflammation. Preoperative drug-induced fibrovascular complex regression brought the possibility to a less bleeding procedure, facilitating the dissection and reducing post-operative hemorrhage. In conclusion, new surgical techniques and pharmacological agents improved the therapeutic arsenal against this harmful disease.
References

Tissue Plasminogen Activator-Assisted Vitrectomy: Surgical Drainage of Submacular Hemorrhage

Motohiro Kamei · Yasuo Tano
Department of Ophthalmology, Osaka University Graduate School of Medicine, Suita, Japan

Abstract
A thick subretinal hemorrhage involving the macula is currently treated with pneumatic displacement or surgical removal. We evaluated the long-term effects of tPA-assisted surgical drainage of a submacular hemorrhage by reviewing the medical records of 12 eyes with submacular hemorrhage followed for a mean follow-up time of 6.9 years. The final best corrected visual acuity (BCVA) improved in 10 eyes (83%), was unchanged in 2 (17%), and was reduced in 0 (0%). The mean preoperative BCVA was 6/200, and the best postoperative BCVA was 20/47, and the final BCVA was 20/143. The late postoperative complications, including recurrent submacular hemorrhage, enlargement of the CNV, and retinal degeneration, reduced the final BCVA. These results indicate that tPA-assisted surgical removal of a subretinal hemorrhage can lead to improved long-term vision. Controlling the choroidal neovascularization may be important for retaining the best postoperative vision.

The visual prognosis of eyes with a submacular hemorrhage associated with age-related macular degeneration (AMD) is generally poor especially when the clot is thick and large [1]. The permanent visual loss after a subretinal hemorrhage is due to photoreceptor damage caused by metabolic interference, iron toxicity, and the shearing of the photoreceptors by a contraction of the fibrin. Experimental studies have shown that the fibrin-mediated damage to the outer retina occurs within 24 h of the onset [2]. Therefore, a thick subretinal hemorrhage involving the macula is currently treated with pneumatic displacement or surgical removal as soon as possible. Mechanical removal or displacement, however, may cause a shearing of the photoreceptors because fibrin interdigitates between the hemorrhagic clot and the photoreceptors.

To overcome this problem, a simultaneous use of tissue plasminogen activator (tPA) is therefore recommended. tPA is a recombinant serine protease which lyses serine
residues in plasminogen and activates it to plasmin resulting in fibrinolysis. Although several studies have reported on the short-term efficacy of tPA-assisted surgical drainage of submacular hemorrhage [3–8], there is no report on the long-term results.

We report on the visual outcome of 12 patients who underwent tPA-assisted surgical drainage of submacular hemorrhage associated with AMD and were followed for more than 5 years after the surgery.

**Patients and Methods**

**Patients**
We reviewed the medical records of 37 eyes with submacular hemorrhage that had undergone tPA-assisted surgical drainage at Osaka National Hospital from July 1992 to October 1996. From the 37 eyes, we selected 12 eyes which were followed for more than 5 year postoperatively. The preoperative opthalmic examination included measurements of the visual acuity and intraocular pressure, slit-lamp and indirect opthalmoscopic examination, visual field analyses, and fluorescein angiography.

**Surgical Procedures**
A standard three-port pars plana vitrectomy was performed, and the posterior hyaloid membrane was carefully separated from the retina. A bent tip 27- or 30-gauge needle (blunt or sharp) was inserted into the subretinal hemorrhage at its superotemporal edge without prior retinotomy or endodiathermy. Then 25 μg/0.1 ml of tPA was slowly injected until it covered the submacular hemorrhage. The total volume of injected tPA ranged from 0.05 and 0.2 ml, which corresponds 12.5–50 μg, depending on the volume of the subretinal blood. After waiting 40–60 min for fibrinolysis to occur, the injection site was enlarged to approximately 1/2 disc diameter by retinotomy with vitreoretinal scissors. Perfluorocarbon liquid was injected onto the inferonasal retina until it reached the retinotomy. The liquefied subretinal hemorrhage was then evacuated into the vitreous cavity through the retinotomy and aspirated with a silicone-tipped back flush needle. A rocking motion to the eye was often used to squeeze the subretinal hemorrhage. Balanced saline solution was injected slowly into the subretinal space through the retinotomy in order to dilute and flush out the fibrin degradation products (FDPs) which were generated from fibrinolysis by plasmin activated by tPA. The FDPs were removed because they have strong chemotactic and inflammatory properties. After a second administration of liquid perfluorocarbon and manipulations to squeeze out the FDPs, a fluid-air exchange was performed. The operation was concluded with infusion of 20% SF6 gas.

**Examinations**
Postoperatively, the patients underwent the same examination as preoperatively under the same conditions. The best-corrected visual acuity (BCVA) was examined by technicians who were masked to the surgical procedures. Changes of the BCVA were assessed according to the postoperative times. Postoperative complications were also recorded and evaluated.

**Results**
There were 8 men and 4 women whose average age at the time of surgery was 62.2 years. The follow-up period ranged from 5 to 10 years (mean: 6.9 years). The final
visual acuity improved by 2 or more lines from the preoperative vision in 10 eyes (83%), was unchanged in 2 (17%), and was reduced in none. Three eyes (25%) had a final BCVA of ≥20/30, 4 eyes (33%) had 20/200 to 20/40, and 5 eyes (42%) had <20/200 visual acuity. The mean preoperative BCVA was 6/200, the postoperative best BCVA was 20/47, and the final BCVA was 20/143.

The early postoperative complications included retinal detachment in one eye (8%) and progression of cataract in 9 eyes (75%). The vision in the eyes with these complications recovered following surgical management. The late postoperative complications included recurrent submacular hemorrhage in 5 eyes (42%), CNV enlargement in 4 eyes (33%), epiretinal membrane formation in 3 eyes (25%), and progression of chorioretinal atrophy in 2 eyes (17%). These late postoperative complications reduced the postoperative BCVA. The postoperative BCVA was maintained in only 4 eyes (33%). Time courses of visual acuity in representative cases are shown in figures 1–4.

Discussion

Bennett et al. [1] reported that the mean final visual acuity in eyes with a submacular hemorrhage was 20/1,700. In another clinical study describing the natural history of submacular hemorrhage associated with AMD, retinal arterial macroaneurysms, and ocular histoplasmosis syndrome, the percentage of eyes that had a significant visual recovery was <50% [9]. Compared to the natural course of a submacular hemorrhage, our results in our 12 patients were better, i.e. they had a mean final visual acuity of 20/143 at almost 7 years after the surgery and a visual recovery rate of 83%.

A number of studies on the short-term results of surgically removing a submacular hemorrhage reported a visual improvement in >70% of the eyes [3–8, 11]. As best we know, there is no report on the long-term results of the visual outcome after a surgical removal of a submacular hemorrhage. Our results demonstrated that tPA-assisted surgical removal of submacular hemorrhage had a long-term effect with an average of approximately 7 years. The long-term results in our patients showed that the visual acuity improved in 83% and the final visual acuity was 20/200 or better in 58%. These findings are comparable to our earlier study of the short-term results where we found that the visual acuity improved in 72 of 92 eyes (78%) and 56% of the eyes had a final visual acuity of ≥20/200.

The surgical removal of a subretinal hemorrhage by forceps was first reported by Hanscom and Diddie [10] in 1987. The mechanical removal of a clot was associated with poor visual results probably because the fibrin interdigitates between the hemorrhagic clot and the photoreceptors, and the removal of the clot resulted in significant damage to the outer retina. Experimental studies have shown that the fibrin-mediated irreversible damage to the outer retina occurs within 24 h of onset [2]. The use of pharmacological agents to release fibrin from the photoreceptor outer segments
Fig. 1. Series of fundus photographs of a representative case (71-year-old man). Thick submacular hemorrhage (a) decreased the vision to counting fingers which improved to 20/25 with tPA-assisted SRH drainage surgery (b: 6 months after the first surgery). Subretinal hemorrhage recurred at 1.5 years after the first surgery and decreased his vision to 4/200 (c). The subretinal hemorrhage was successfully removed again at second surgery and vision recovered to 20/30 (d). Vision has been maintained for more than 4 years after the last surgery (e).
Fig. 2. Time courses of visual acuity in representative cases. 

a Case 1: a 63-year-old man. His cataract worsened in the 3 months after the surgery, resulting in a decrease of vision. His vision improved up to 20/25 after cataract surgery and was maintained for more than 9 years after the first surgical removal of a submacular hemorrhage.

b Case 2. a 70-year-old woman. Her best visual acuity improved to 20/40 immediately after the surgery, but cataract progression and CNV enlargement depressed her vision. Cataract surgery 1 year after the vitrectomy partly improved the vision, but unfortunately remained low at 18/200.

c Case 3: a 76-year-old man. Cataract progressed 2 years after the surgery resulting in a decrease of vision. His vision improved up to 20/25 after cataract surgery, but a recurrence of a submacular hemorrhage occurred 3 years after the surgery and the visual acuity decreased to 2/200. The second surgical removal of submacular hemorrhage was performed and the vision recovered up to 20/100, which was maintained for 5 years after the last surgery.

d Case 4: a 74-year-old woman. A good visual acuity of 20/40 was maintained for 2 years with cataract surgery. However, chorioretinal atrophy gradually progressed and decreased the vision to 6/200.
appears reasonable and necessary. Subretinal injections of tPA have been shown to liquefy submacular hemorrhages, and the liquefied clots are evacuated through a small retinotomy, which reduced surgery-induced damage to the neural retina and retinal pigment epithelium.

The early postoperative complications of surgical removal of submacular hemorrhage in the previous reports [3–8] included disciform scar formation due to persistent choroidal neovascularization (11%), recurrence of the hemorrhage (5–18%), retinal detachment and PVR (4–14%), epiretinal membrane formation (14%), and cataract progression (12–53%). In our patients, we found retinal detachment in one eye (8%) and progression of cataract in 9 eyes (75%) as early postoperative complications, and recurrent submacular hemorrhage in 5 eyes (42%), CNV enlargement in 4 eyes (33%), epiretinal membrane formation in 3 eyes (25%), and progression of chorioretinal atrophy in 2 eyes (17%) as late postoperative complications. Because these late postoperative complications depressed the postoperative BCVA, suppressing CNV activity seems important for maintaining the better vision.

**Current Indications for tPA-Assisted Surgical Removal of Submacular Hemorrhage**

Although the first choice for treating subretinal hemorrhage is currently pneumatic displacement, we demonstrated in this study that tPA-assisted surgical removal can lead to improved long-term vision. We are currently using tPA-assisted surgical removal of submacular hemorrhage in the following cases: submacular hemorrhage that lasts less than 2 weeks or appears predominantly red with minimal yellowish-white color, ≥500 μm thick, and ≥2 disc area in size. Other factors, such as conditions of the opposite eye, systemic conditions, and visual acuity immediately before the submacular hemorrhage developed, should be considered for selecting tPA.

**References**

Anti-Angiogenic Therapy in the Management of Retinopathy of Prematurity

Kimberly A. Drenser

Associated Retinal Consultants, Royal Oak, Mich., and Eye Research Institute, Oakland University, Rochester, Mich., USA

Abstract

Retinopathy of prematurity (ROP) is a vitreoretinal abnormality that significantly affects premature babies with low birth rates. Despite improved screening and management of these infants, a subset will progress to retinal detachment and permanent visual impairment. Current treatment consists of peripheral laser ablation and subsequent surgical intervention if a detachment occurs. We sought to evaluate the vitreous biochemistry of eyes that progress despite appropriate laser intervention. Additionally, a limited trial of an anti-VEGF (vascular endothelial growth factor) therapy was used in one eye of infants with persistent Plus disease and neovascularization. The anti-VEGF treatment successfully decreases abnormal angiogenesis but does not decrease the proliferative changes associated with retinal detachment. Biochemical analysis of the vitreous of stage 4 ROP eyes shows significantly elevated VEGF and transforming growth factor (TGF-β) concentrations, and normal levels of other angiogenic factors.

Background

Retinopathy of prematurity (ROP) continues to be a leading cause of blindness in children in developed countries around the world, and an increasing cause of blindness in developing countries. The current standard of treatment is ablation of the peripheral avascular retina. Screening and intervention is now directed by the criteria established by the ETROP Study [1]. The ETROP Study demonstrated superior results compared to the Cryo-ROP Study but also recommends earlier treatment with laser ablation. In the children requiring laser treatment the peripheral retina is ablated and destroyed for future use. The ablated retina is not functional and is not amenable to regeneration. In addition, morbidity from laser can include cataract and vitreous hemorrhage as well as anterior segment ischemia, which can lead to phthisis bulbi.

The mechanism driving the development of ROP is in large part dependent on vascular endothelial growth factor (VEGF) [2–5]. The normal biochemistry of the
developing eye is altered due to the change in environment when a baby is premature. During the second trimester the developing fetus enters phase 1 of vascular development. During phase 1 the endogenous VEGF levels of the fetus are elevated to promote angiogenesis and vascular maturation. In contrast, the relatively hyperoxic environment that the premature baby is introduced in to results in the converse reaction, with decreased production of VEGF leading to delayed retinal maturation. Phase 2 occurs during the third trimester and during this phase the developing fetus will normally reduce VEGF production [6]. VEGF production becomes dysregulated in the premature child, presumably due to large areas of avascular retina creating tissue hypoxia, resulting in abnormally high levels of VEGF. This biochemical shift heralds the pathological changes seen in ROP and explains why the peak incidence of stage 3 ROP occurs between a postmenstrual age of 36–38 weeks [3].

Recently, a subset of patients developing a severe form of ROP has been identified [1]. This group develops an aggressive form of zone 1 disease, designated as aggressive posterior ROP (APROP). APROP develops between postmenstrual age (PMA) 32–34 weeks and is seen in very low birth weight infants generally with a GA less than 26 weeks. It has the poorest outcome with many eyes progressing to retinal detachment despite complete and timely laser ablation of the avascular retina [7].

Vitreous Biochemistry

Vitreous samples were taken at the time of surgery for stage 4 ROP eyes (± pegaptinib treatment). The fluid was analyzed by ELISA assay for total VEGF, TGF-β (isomers 1 and 2), hepatocyte growth factor (HGF) and insulin growth factor (IGF-1). We found significantly elevated levels of VEGF and TGF-β in eyes with ROP-related retinal detachment (fig. 1). The average age of surgery was postmenstrual age 42 weeks, a time point that represents the most frequent onset of tractional retinal detachment in ROP eyes that have undergone laser ablation. Vitreous VEGF concentrations were 576 pg/mg protein in vascularly active eyes and 56 pg/mg protein in nonactive ROP eyes, compared to 12 pg/mg protein in control eyes. The VEGF was most elevated in eyes that demonstrated persistent vascular activity characterized as Plus disease or neovascularization at the time of surgery [2]. It is interesting that despite appropriate and timely laser ablation elevated levels of VEGF persist in the ROP eyes. This finding indicates that the biochemical disturbance is not due to avascular retina alone.

There are a number of other angiogenic factors that have been associated with neovascularization in various eye diseases. HGF and IGF-1 have been associated with proliferative diabetic retinopathy [8–10], although there have been discrepancies regarding the intravitreal HGF levels [11]. We evaluated the vitreous samples for other factors implicated in angiogenesis (HGF and IGF-1) and found that they were not elevated in eyes with ROP compared to controls. From these studies, it appears that VEGF is the primary factor driving Plus disease and neovascularization in ROP.
Transforming growth factor has an anti-angiogenic activity and also increases pre-retinal proliferative changes. It has been noted that TGF-β levels increase near the due date of premature infants and may play a role in the final suppression of intraocular angiogenesis. The majority of infants with ROP requiring laser ablation do not go on to develop a retinal detachment. Interestingly, those infants that do develop a tractional retinal detachment following appropriate laser ablation do so shortly after their due date (highest incidence at PMA 42 weeks). The vitreous samples of stage 4 ROP eyes were evaluated for the concentrations of the two predominant intraocular isomers of TGF-β (1 and 2). TGF-β2 is the predominant form, accounting for nearly 90% of the total TGF-β. Although TGF-β1 only accounts for approximately 10%, it may play a greater role in proliferative events due to its control of pericyte growth [12]. TGF-β1 averaged 145 pg/mg protein in ROP eyes compared to 4 pg/mg protein in control eyes. TGF-β2 averaged 1,744 pg/mg protein in ROP eyes compared to 470 pg/mg protein in control eyes. When compared to controls these are significantly higher concentrations. However, the TGF-β levels for both isoforms are also significantly higher than those seen in rhegmatogenous retinal detachment and proliferative diabetic retinopathy (fig. 2, 3). This finding supports the suggestion that TGF-β levels increase with increasing elevations of VEGF and likely accounts for the small subset of ROP eyes that progress to tractional retinal detachment despite timely and complete laser ablation of the avascular retina.

**Anti-Angiogenesis Treatment**

With the advent of FDA approved drugs for anti-VEGF treatment, the possibility of treating ROP with anti-VEGF agents has become possible. Drugs that are available...
include the drug pegaptinib (Macugen) for partial blockage of VEGF-A, or complete blockage of VEGF-A with drugs such as ranibizumab (Lucentis) and bevacizumab (Avastin). Some clinicians have evaluated the usefulness of bevacizumab (Avastin) in ROP. These were retrospective case reports or series. All reports demonstrated safety and tolerance of an intravitreal anti-VEGF treatment and all investigators found a rapid resolution of Plus disease and/or neovascularization [13, 14]. One report, however, did find severe progression of fibrosis and retinal detachment with a funnel detachment ensuing [15]. Of note, this particular case received the treatment when proliferative changes were noted and an early detachment was already occurring. Many clinicians have seen similar tractional changes following anti-VEGF treatment of patients with proliferative diabetic retinopathy [16, 17].

In a prospective limited trial, our institution evaluated the effect of partial VEGF inhibition using pegaptinib. VEGF is required in the developing retina for normal angiogenesis and neurogenesis [4] and it was felt that partial VEGF blockade may be beneficial to the developing retina and its vasculature. Pegaptinib was developed to inhibit the VEGF_{165} isomer only, with the theory that this particular isomer represents the pathological changes seen with increased VEGF concentrations. The goal of intravitreal anti-VEGF treatment was to block the excessive levels of VEGF trapped within the overlying vitreous and maintain a normal vanguard of intraretinal VEGF, promoting continued vascular maturation. Eyes requiring surgery had biochemical analysis of their vitreous fluid in order to evaluate other potentially important agents in the development of ROP and progression of retinal detachment.
Premature babies with zone 1 ROP (fig. 4) requiring treatment per ETROP criteria underwent standard care using peripheral laser ablation. Infants with zone 1 disease who demonstrated continued vascular abnormalities (persistent Plus disease or neovascularization) despite appropriate and complete laser ablation and were less than 36 weeks of gestational age were offered rescue therapy with pegaptinib. A total of 5 infants participated and each infant had one eye treated with intravitreal anti-VEGF agent. The study eye received two injections of 1/2 dose pegaptinib (0.05 ml) 1 week apart. The fellow eye was managed by standard care with laser ablation and surgical intervention if progression to tractional retinal detachment was noted. Dilated fundus examination with fundus photography was performed weekly. Surgery was performed if a tractional retinal detachment developed in either eye.

Three infants progressed to bilateral retinal detachment despite treatment (laser ablation of both eyes; one eye received pegaptinib). Of note, all of the pegaptinib-treated eyes demonstrated prompt resolution of Plus disease and regression of the tunica vasculosa lentis 1 week after the initial intravitreal injection (fig. 5) (1/2 dose), consistent with the findings of other investigators. The eyes remained quiet for the remainder of the study. Also, of interest, the pegaptinib-treated eyes that went on to tractional retinal detachment developed the detachment 1–2 weeks after the laser – only eye developed detachment, indicating that decreasing vascular permeability delays the onset of retinal detachment. Once retinal detachment began, however,
The pegaptinib-treated eyes had an aggressive fibrosis and proliferation that led to an accelerated detachment progression (fig. 6). Despite the anti-VEGF treatment not preventing retinal detachment, early surgical intervention at the first sign of fibrosis and/or retinal detachment leads to an improved long-term result, both structurally and functionally, compared to the laser-only fellow eye (fig. 7).

The two pegaptinib-treated eyes that did not progress to tractional retinal detachment received the first intravitreal injection at the time of laser, and therefore, at the youngest age (post-menstrual age 33 weeks) in this study group. Presumably, this early anti-VEGF inhibition results in early reversal of the pathologic effects of VEGF accumulation in the vitreous and stabilizes the intraocular biochemistry. Blockage of VEGF accumulation when the fetus is entering phase 2 may improve the anti-VEGF treatment response and reverse the pathological angiogenesis.

**Fig. 4.** a Persistent tunica vasculosa lentis with poor pupil dilation. b Zone 1, stage 3, early Plus. Both eyes had symmetrical disease.

**Fig. 5.** Zone 1, stage 3, early Plus OU; 1 week after treatment. a Right eye: treated with laser ablation and pegaptinib. Note resolution of Plus disease. b Left eye: treated with laser only. Note persistent Plus disease.
As noted previously, it has been suggested that TGF-β levels endogenously upregulate near the due date and that the level of TGF-β corresponds to the levels of VEGF present at that time. Binding of VEGF does not appear to alter the concentration or effects of TGF-β. TGF-β plays a role in proliferative changes affecting the retina and the rapid binding of VEGF may allow unopposed TGF-β to have a more dramatic proliferative response. Support of this theory is seen in the vitreous sample of infants with stage 4 ROP, where the levels of TGF-β are elevated significantly above those seen in other proliferative diseases (fig. 2, 3). Additionally, the intravitreal concentrations of TGF-β are greatly elevated compared to the VEGF concentrations in eyes with stage 4 ROP at the time of surgery (fig. 1). These findings indicate that early VEGF inhibition is key to successful anti-VEGF treatment in ROP.

**Fig. 6.** Postmenstrual age 42 weeks; bilateral retinal detachment. **a** Right eye: early nasal retinal detachment with posterior pole distortion s/p pegaptinib. **b** Left eye: extensive nasal retinal detachment with folding of the temporal retina.

**Fig. 7.** 2 months s/p vitrectomy OU. **a** Right eye: excellent anatomical and visual outcome (20/40). **b** Left eye: poor anatomical outcome with light perception only.
**Conclusion**

The biochemical constituents of patients with stage 4 ROP indicate that abnormally high levels of VEGF and TGF-β are present and appear to be the predominant factors driving pathological angiogenesis and retinal detachment. HGF and IGF-1 have been implicated in angiogenesis, especially in diabetic patients [9, 10, 18]. HGF has also been implicated in neuronal differentiation [11, 19] and is lower than expected in these samples. Both HGF and IGF-1 are expressed at low levels and do not appear to play a significant role in ROP pathology. The surprisingly low levels of IGF-1 in the vitreous of ROP infants may be an indication of delayed infant maturation or further biochemical abnormalities in the premature eye.

Inhibition of VEGF effectively decreases the vascular pathology and results in stabilization of the vasculature [4]. It does not, however, prevent the progression to retinal detachment. The significantly elevated levels of TGF-β likely contribute to the tractional changes seen in these eyes. TGF-β may be abnormally elevated as a secondary response to high intravitreal VEGF concentrations. Possibly, the blocked VEGF leads to unopposed TGF-β activity and promotes proliferation of cells and tractional retinal detachment. Additionally, the incomplete regression of primary vasculature and vitreous may provide a platform of increased cellular substrate for TGF-β to act upon, creating greater tractional forces. Earlier intervention with an anti-VEGF agent may avoid this late complication and further studies will need to be conducted to answer this. Prompt surgical intervention, however, results in a favorable outcome. The anti-VEGF treatment quiets the vascular abnormalities and creates less intraoperative bleeding, allowing for early surgical intervention if necessary. Alternatively, a TGF-β inhibitor may be helpful in preventing retinal detachment in these patients.

Continued investigation into the pathology of ROP and the use of biochemical modulation with timely surgical intervention may lead to improved visual outcomes for these infants.

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