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The role of Raf-1 kinase in diabetic retinopathy

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Abstract

Many metabolic pathways, including oxidative stress, PKC and the polyol pathway have been implicated in the development of diabetic retinopathy, but despite extensive research, its pathogenesis remains unclear. Recent studies have shown the role of a low-molecular-weight GTP-binding protein (H-Ras)-mediated signaling pathway in its development. The key effector protein of Ras function is a threonine/serine kinase-Raf kinase, and this kinase is involved in a variety of functions, including the cell cycle and proliferation and apoptosis. In animal models of diabetic retinopathy, Raf kinase is activated in the retina and its microvasculature. Activated Raf kinase is associated with increased apoptosis of retinal capillary cells, the process that precedes the development of retinal histopathology, and inhibition of Raf kinase ameliorates apoptosis. In clinical settings, inhibitors of Raf kinase have shown promising results in cancer treatment, and Raf kinase antisense oligonucleotides, iCo 007, is now in Phase II trial for macular edema, a chronic ocular disease associated with retinal neovascularization. Further elucidating the role of Raf kinase in diabetic retinopathy, and advances in the generation of antisense therapy for chronic diseases, should help test Raf antisense oligonucleotides for the treatment of this blinding complication that diabetic patients fear the most.

Keywords

diabetes; diabetic retinopathy; Raf kinase; retina

1. Diabetic retinopathy

Diabetic retinopathy, a slow progressing and multifactorial complication of diabetes, is the most common cause of vision loss in diabetic patients. The development of retinopathy is directly related to the duration of diabetes, it is rarely detected in the first few years of diabetes, but by 10 years, approximately 50% of the patients, and by 20 -- 25 years, nearly 90% of the diabetic patients present some stage of retinopathy [1,2]. There are no early warning signs that accompany diabetic retinopathy and in the early stages of the disease vision is not affected. But, when visual problems start to appear, retinopathy

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has already advanced, almost a point of no return. It is a highly specific vascular disease in which damage to the small blood vessels of the eye can lead to blindness. Diabetic retinopathy is characterized by capillary microaneurysms, cotton-wool spots, hemorrhage, exudates and the formation of highly permeable new vessels [3,4]. The Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study have demonstrated that glycemic and blood pressure control can prevent and delay the progression of diabetic retinopathy in patients with diabetes [5,6]. However, since it is difficult to maintain blood glucose concentrations close to the normal range for this lifelong disease, present treatment options are limited to somewhat invasive options such as photocoagulation and intraocular injections [4], thus, identification of therapeutic targets to halt or slow down the progression of this blinding disease based on the molecular mechanism of its development is essential.

One of earliest changes observed during the development of retinopathy in diabetes is the leakage of retinal vessels into the surrounding tissue, and thickening of the basement membranes. Retinal capillary cells and other retinal cells start to undergo accelerated apoptosis, and this precedes the detection of any histopathological changes characteristic of diabetic retinopathy [7–10]. Retinal capillaries become degenerative and the surrounding pericytes are lost, and with time, this results in ischemia and release of angiogenic growth factors [11–14]. Over the last several years, studies have explored the involvement of various metabolic pathways, including PKC, low-molecular-weight GTPbinding proteins (G-proteins) and MAPK, oxidative stress and endoplasmic stress mediated signaling pathways in the pathogenesis of diabetic retinopathy [15–21]. Understanding of such mechanistic pathways is critical for developing future adjuvant therapies for diabetic retinopathy. The focus of this review is on the role of Raf-1 in diabetic retinopathy.

2. Ras--Raf pathway

One of the biological switches for cellular processes is a group of low-molecular-weight GTP-binding proteins, and the protein subfamily that is involved in cellular signal transduction is the subfamily of homologous small molecular GTPbinding proteins consisting of H-, N- and K-Ras [22]. The most ubiquitous among three is H-Ras (referred as Ras throughout this article), a 21-kDa membrane-associated monomeric GTPase, which cycles between a GTP-bound active and a GDP-bound inactive state [22]. It converts signals from the cell membrane to the nucleus and stimulates a critical network of signal transduction pathways that leads to cellular proliferation, survival and differentiation. Activation of Ras in selective compartments is considered to have distinct physiological outcomes [22,23]. Ras mediates its actions through activation of multiple downstream effector signaling cascades, which in turn regulate transcription factor activation and cause changes in gene expression.

A key effector protein of Ras function is a threonine/serine kinase, c-Raf. Raf kinases are a family of three kinases that are related to retoviral oncogenes, and are involved in a variety of functions, including cell cycle and proliferation and apoptosis [24]. C-Raf (referred to as Raf in this article) binds efficiently to GTP-bound Ras (activated form), and this binding activates Raf. Activated Raf subsequently phosphorylates MAPK kinase 1/2 (MEK 1/2),

which then phosphorylate their two known substrates, extracellularsignal-regulated kinases 1/2 (ERK 1/2) [22]. ERK1/2 kinases phosphorylate a variety of downstream targets resulting in changes in gene expression and the catalytic activities of various enzymes [25]. Thus, Raf-MEK-ERK kinase cascade is a fundamental component of both normal and pathological cell regulatory networks, and Raf kinase is an integral part of the Ras/Raf/MEK/ERK kinase cascade.

3. Regulation of Raf kinase

Raf kinase, in its dormant form, is mostly present in the cytosol and activated Ras recruits it to the plasma membrane. In physiological settings, regulation of Raf kinase is achieved by many different mechanisms, including protein--protein interactions, phosphorylation and conformational changes [26]. Raf kinase inhibitory protein (RKIP) also modulates Raf signaling pathway by forming ternary complexes with Raf, MEK and ERK, however, RKIP can also dissociate a Raf-1--MEK complex and behave as a competitive inhibitor of MEK [27]. Another regulator of Raf kinase, Raf kinase trapping to Golgi (RKTG), binds to Raf and translocates it into the Golgi apparatus, and Raf activation reduces association of Raf with Ras and MEK and blocks the ERK pathway. In renal cell carcinoma, RKTG is shown to inhibit angiogenesis by suppressing MAPK-mediated autocrine VEGF signaling [28].

One of the other mechanisms via which Raf kinase is regulated is via oxidative stress. Studies have shown that increased reactive oxygen species often activate the Raf -- MEK -- ERK cascade through Ras, leading to cellular dysfunction/death [29,30]. However, in many cell types, sustained expression of activated Ras/Raf, elicits cell cycle arrest or senescence, providing a defense mechanism against inappropriate activation of the Ras/Raf signal transduction pathway. Conversely, Ras/Raf complex is also involved in the signaling steps leading to the induction of intracellular oxidative stress, and interactions between Ras/Raf and several of its effector proteins are affected by reactive oxygen species [29,31–33].

Early studies of Ras signal transduction have suggested a linear signal transduction cascade of Ras--Raf--MEK--ERK, however, it has become increasingly clear that Ras can mediate its actions through interactions with both Rafdependent and Raf-independent effectors [34]. Additionally, there is accumulating evidence that components of the individual linear pathways also engage in cross talk. Persistent activation of Raf also activates NF-κB, which can upregulate the expression of several downstream factors associated with inflammation [35]. Thus, Raf kinase has an integral role in a wide range of cellular functions and the cell is equipped with multiple facets to maintain its homeostasis.

4. Raf kinase and diseases

Since Raf has a central role in many signaling pathways by connecting upstream tyrosine kinases with downstream serine/threonine kinases, aberrant signaling of the Ras--Raf--MAPK cascade has been implicated in a variety of human diseases like melanoma, polycystic kidney, cancer, diabetes, Alzheimer, inflammatory and autoimmune diseases (rheumatoid arthritis, Crohn’s disease, psoriasis). In diabetes, Ras/Raf signaling

is implicated in the upregulation and overproduction of physiological inhibitor for fibrinolysis associated with atherothrombotic cardiovascular diseases [36,37]. Glucose-mediated regulation of the Raf/ERK signaling pathway is associated with secretion of insulin [38], and these kinases are also involved in the diabetic embryopathy [39]. However, the role of Raf kinase in diabetic retinopathy remains a sparingly tested area.

5. Raf kinase and diabetic retinopathy

Recent results have shown that the pathways activated by Ras/Raf play a crucial role in diabetes-associated complications including retinopathy. Raf is activated in the retina and its microvasculature in animal models of diabetic retinopathy, and increased apoptosis of retinal capillary cells in diabetes, which precedes the development of histopathology, is, in part, mediated via activation of Raf pathway. Further, therapies that prevent the accelerated apoptosis of retinal capillary cells and the development of diabetic retinopathy in rats also inhibit retinal Raf activation [10,16,18,21,30,40–42]. Ras/Raf activation is required for the pro-angiogenic response to VEGF [28], and VEGF is one of the major mediators in intraocular neovascularization and plays a pivotal role in the pathogenesis of diabetic retinopathy [43]. The activation of Ras/Raf is also associated with pro-apoptotic signaling and this is dependent on the cell type and the environment [44,45]. A growing body of evidence from various cells, including retinal endothelial cells, suggests that activation of the Ras/Raf signaling pathway leads to cell apoptosis [40,46–48]. ERK activation, generally considered a survival pathway, has also been shown to be pro-apoptotic, possibly through death-promoting targets such as B-cell leukaemia/lymphoma-associated X protein (Bax) and apoptotic peptidase activating factor 1 (Apaf-1) [49], and in diabetes, capillary cell mitochondria are leaky with cytochrome moving out of the mitochondria and Bax translocating into the mitochondria [50]. In addition, inhibition of ERK activation attenuates apoptosis of various cells [51–53]. ERK phosphorylation downregulates GAP junction communication via connexin 43 phosphorylation [54], and connexin-43 is considered to play an important role in the apoptosis of retinal capillary cells in diabetes [55]. ERK activation has been shown to promote apoptosis of brain endothelial cells [56]. In retinal endothelial cells inhibition of Raf kinase prevents glucose-induced activation of NF-κB [16,18,21], and the development of diabetic retinopathy. Although NF-κB is considered to be an anti-apoptotic transcriptional factor, in the pathogenesis of diabetic retinopathy it has been shown to act as a promoter of apoptosis in retinal capillary cells; [57–59]. Furthermore, activation of retinal NF-κB is an early event that can be prevented by therapies that inhibit the development of diabetic retinopathy in rats [58], and NF-κB inhibition decreases diabetes-induced degeneration of retinal capillaries [60]. This implies that the Ras--Raf--MEK--ERK signaling cascade functions as a pro-apoptotic pathway, and apoptosis of retinal capillary cells is considered a surrogate marker of pathology associated with diabetic retinopathy [9].

6. Raf kinase inhibitors

As stated above, the Ras--Raf--MEK--ERK cascade participates in the regulation of a wide variety of processes including apoptosis, cell cycle progression, differentiation and proliferation and thus this cascade has become an attractive therapeutic target for several diseases. Inhibitors of Raf kinase have shown promising results in experimental and clinical

cancer therapy. Several selective Raf and MEK small-molecule inhibitors are being tested in Phase I and Phase II clinical trials. These inhibitors prevent the expression of Raf protein by blocking Ras--Raf interaction or obstructing its kinase activity. Sorafenib (BAY 43 -- 9006), a Raf kinase/VEGF-R2 inhibitor has already been approved for treatment of renal cancer and is being tested for breast cancer [61]. It also inhibits the proliferation in tumor cell lines, and diminishes the replication of human cytomegalovirus, a major pathogen in immunocompromised individuals [62]. In addition, Sorafenib has recently entered Phase III clinical testing with promising signs of anti-cancer efficacy, and a very tolerable safety profile. It suppresses ERK phosphorylation, and dephosphorylates B cell leukemia/lymphoma associated protein 2-associated agonist of cell death (Bad), activating B cell leukemia/lymphoma associated protein 2-antagonist/killer 1(Bak) and Bax and reduces the mitochondrial transmembrane potential. Raf kinase antisense oligonucleotides ISIS 5132 and LeRafAON, Raf destabilizers geldanamycin, and Ras--Raf interaction inhibitor monocyte chemotactic protein-1 (MCP-1), are currently being used in clinical trials [63].

7. Raf-kinase inhibition and diabetic retinopathy

Retinal capillary loss is an early event in the pathogenesis of diabetic retinopathy leading to retinal neovascularization, the major cause of blindness in diabetic patients. A great deal of attention has been focused on the role of VEGF [64], but since diabetic retinopathy is a duration-dependent slowly progressing disease, its inhibition in the early stages before neovascularization starts to appear, is critical. Understanding the molecular mechanism of the development of the disease is critical in identifying the therapeutic targets. Animal data, though limited, strongly suggest that activation of Raf kinase is associated with the development of diabetic retinopathy [16,18,21]. Although Raf activation is considered to be upstream of VEGF activation, VEGF has been reported to activate Raf kinase in human endothelial cells [65], suggesting a possible autocrine loop. We have shown that activation of Raf kinase accelerates the loss of retinal capillary cells in response to elevated glucose. Inhibition of Raf kinase by GW5074 prevents retinal endothelial cells from glucose-induced apoptosis, and prevents activation of NF-κB, a transcriptional factor with an important role in the development of diabetic retinopathy [18]. Various growth factors and cytokines (e.g., VEGF, IL-1B and intracellular adhesion molecule-1) are considered to be downstream of the Raf -- MEK signaling cascade [66,67], and regulation of Raf, a target which is upstream of these growth factors and cytokines, should be more effective than targeting individual growth factors. In addition, as mentioned above, the effect of Sorafenib on mitochondrial potential raises the possibility that a Raf kinase inhibitor could also inhibit diabetic retinopathy via mitochondrial pathways because mitochondrial dysfunction is considered to play an important role in the pathogenesis of diabetic retinopathy [50]. Thus, there is an attractive possibility that the Raf inhibitors have a great potential to inhibit the development of retinopathy in diabetes, both in early stages via inhibiting capillary cell apoptosis and in later stages via inhibiting neovascularization.

8. Antisense oligonucleotide approach to treating diseases

Oligonucleotides have become one of the new treatment strategies in the disease process. Antisense oligonucleotides are single-stranded DNA molecules with sequences

complementary to specific mRNAs. They function via inhibiting the expression of specific genes. The therapy is based on the use of approximately 20 nucleotides (oligonucleotide) synthesized to be complementary to the specific 'sense' (5′ to 3′ orientation) mRNA sequence responsible for coding of the targeted protein [68]. Antisense drugs function via different mechanisms; they can inhibit the splicing and mRNA maturation or inhibit ribosomal readthrough and interrupt the translation phase of the protein production process by preventing the mRNA instructions from reaching the ribosome. Many studies have used the properties of antisense oligonucleotides to inhibit gene expression in cultured cells, and, though limited, their use has also been extended to whole organisms. These therapies are relatively sensitive, and if the gene sequence relevant to the patient is identified, these nucleotides can be synthesized within a very short frame of time at relatively lower cost. Another major advantage is that their binding action can be controlled, measured for reactions or responses, and then redirected as necessary. With the discovery of the second-generation antisense oligonuclotides, antisense drugs are being researched to treat cancers, diabetes and diseases such as asthma and arthritis with an inflammatory component. However, these therapies can stimulate the immune system and decrease the effectiveness of the therapy, the vectors could produce toxicity and inflammatory responses and there is a possibility of insertional mutagenesis [69]; these shortcomings need to be considered.

The recent availability of animal models of diabetes has provided an opportunity to use antisense oligonucleotides to understand the mechanisms of disease pathology and to potentially intervene therapeutically [70,71]. There are a number of antisense oligonucleotides being used for diabetes; ISIS 113715 is an insulin sensitizer that reduces the expression of protein tyrosine phosphatase-1B. In Phase II clinical trials, it has shown promising results by lowering blood glucose in patients with type 2 diabetes. ISIS 325568, an antisense drug designed to inhibit production of the glucagon receptor, improves blood glucose in patients with type 2 diabetes. ISIS-377131 inhibits glucocorticoid signaling selectively in the liver and fat tissue, and improves blood glucose levels. iCo 007, an antisense inhibitor of c-Raf kinase, is in Phase II trial for macular edema. This oligonucleotide reduces the formation and leakage of new blood vessels in the eye. Thus, though in the early stages, oligonucleotides have shown promising results for chronic diseases. Despite exciting discoveries, many uncertainties remain about the therapeutic potential of synthetic oligonucleotides. The effectiveness and the specificity of the antisense oligonucleotides in biological systems are some of the issues that should be considered. Furthermore, in some tumors, a gene could be activated by a single nucleotide mutation, and the antisense oligomers can selectively inactivate the mutated but not the normal gene [72].

9. Antisense treatment for diabetic retinopathy

Despite extensive research in the diabetic retinopathy field, the use of antisense therapy to inhibit its development remains largely an untested area. Consideration of novel approaches to treating diabetic retinopathy is of great interest because diabetes is becoming the epidemic of 21st century, and with that, the incidence of retinopathy is escalating at an alarming rate.

Oligonucleotides targeted against the translation initiation site of the fibronectin transcript have reduced fibronectin mRNA and protein level, and capillary basement membrane

thickening in galactose-fed rats, another animal model of diabetic retinopathy [73]. Transfection of microvascular endothelial cells with AS-connexin 43 oligonucleotides is shown to inhibit glucose-induced alterations in intercellular communication and maintain vascular homeostasis [55]. Growth hormone receptor antisense oligonucleotide reduces hypoxia-induced retinal neovascularization [74]. These studies, though limited in number, suggest that there is a potential for antisense therapy to produce beneficial effects to inhibit the development of diabetic retinopathy. Thus, the clinical use of the second-generation antisense oligonucleotides, iCo-007 (which targets c-Raf kinase) for macular edema, now approved for Phase II clinical trials (iCo Therapeutics), and our recent results showing the role of Raf kinase in diabetic retinopathy [18,21], opens up the possibility of exploring the use Raf-targeted antisense oligonucleotides for diabetic retinopathy.

However, due to a well established blood--retina barrier, the route of antisense oligonucleotide delivery is an important issue for treatment of diabetic retinopathy. For targeted delivery of antisense oligonucleotide, intravitreal injections (routinely used by ophthalmologists for anti-VEGF treatment) can be utilized. In addition, the intravitreal route has other advantages, including the use of a low drug dose and quick response time. Though the intravitreal route remains the commonly used option for delivery to the posterior of the eye, this is also associated with a range of complications, including the possibility of elevating intraocular pressure, cataracts, retinal detachment and infection, the conditions that a diabetic patient is already at higher risk of getting. Since diabetes is a lifelong disease, long-term safety and repeated intraocular administration are some important points that should be addressed. Periocular means, exploiting the permeability of sclera for retinal drug delivery, including subconjunctival, retrobulbar and peribulbar routes, are particularly useful for administering sustained-release systems of potent drugs. Another viable option for the treatment of this ocular manifestation of a systemic disease is intravenous administration, but the systemic side effects and delivery of sufficient drug to the retina because of a well established blood--retinal barrier could be some of the major shortcomings diminishing the efficacy of the drug. Also, intravenous or topical application with the possibility of absorption of the drug systemically could produce some unwanted side effects associated with synthetic oligonucleotides.

Diabetic retinopathy is a slowly progressing disease, DCCT studies have clearly documented that hyperglycemic insult contributes to its development over a long period of time. Reversing prior effects of poor glycemic control or prior benefits of good glycemic control extend beyond the period of their institution [5,6]. This clearly suggests that the time of initiation of therapy is critical, and to achieve benefits of a therapy, it is important to initiate the therapy at earlier stages. In addition, retinopathy begins to develop at least seven years before the clinical diagnosis of type 2 diabetes [75]. Early detection of retinopathy in patients with diabetes is critical in preventing visual loss, but current methods of screening fail to identify a sizable number of high-risk patients, and the exact time of initiation of any therapy is difficult to pin point. The use of antisense therapy during early stages of the disease, however, should provide greater benefit than when initiated at later stages.

Identification of a good antisense therapy to treat diabetic retinopathy is essential to alleviate the risk of this blinding complication feared the most by diabetic patients. With the ongoing

research in understanding the pathogenesis of diabetic retinopathy, drug delivery methods and improving the specificity of antisense therapy, we strongly believe that antisense therapy could be one of the viable options available to ophthalmologists to treat this devastating disease, which plagues over 80% of patients after 15 -- 20 years of diabetes.

10. Expert opinion

Diabetic retinopathy, the major cause of acquired blindness, remains difficult to prevent and treat. The disease is multifactorial and slow progressing and the mechanism (s) via which it manifest remains unclear. The scientific community is conducting extensive research to understand the pathogenesis, and to elucidate molecular targets for future therapeutics. With the newer biomedical molecular and genetic approaches, there is hope that we will be able to better understand the mechanism of the development of diabetic retinopathy.

The role of Raf kinase in the development of diabetic retinopathy is a novel area. Experimental studies have shown that Raf kinase and the Ras--Raf--MEK--ERK signaling cascade play an important role in the development of diabetic retinopathy. The use of the second-generation Raf kinase targeted antisense oligonucleotides for another chronic ocular disease, macular degeneration, and the recent advances in the drug delivery to the targeted area, opens up the door for future testing of Raf kinase-antisense oligonucleotides for diabetic retinopathy.

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