Cellular and Molecular Mechanisms of Neurodegeneration in Early-Stage Diabetic Retinopathy

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Background

Diabetic retinopathy (DR) remains the leading cause of blindness in working age Americans. There has yet to be any effective treatment to prevent the onset of the condition, only to treat late-stage disease. Research on early signs of disease have shown that changes in neural layers of the retina are the earliest signs of disease, preceding the vascular changes that currently define DR. This has sparked interest in the pathogenesis of the neurodegeneration involved in DR. This review explains the current understanding of the cellular and molecular mechanisms of neuronal degeneration in DR, as well as the potential pharmacological interventions that are being researched for each mechanism.

Methods

A literature review was performed to look at each major cellular and molecular pathway that has been defined and associated with DR-related neurodegeneration, the most current research regarding pharmacological interventions, and the relationship between the retinal neural cells and the microvasculature in diabetes to promote neurodegeneration. Articles have been sourced from either PubMed or Up-To-Date.

Results

The polyol, PKC, hexosamine, and AGEs pathways have been shown to be upregulated in hyperglycemia. The polyol pathway descreases NADPH, which is necessary for glutathione regeneration. Neural cells become unable to tolerate ROS. Fructose and sorbitol accumulate in cells, causing swelling. Epalrestat, FDA approved for diabetic neuropathy to target aldose reductase, has potential for DR. The PKC and RAGEs pathways promotes NADPH oxidase which produces ROS. PKC-β inhibitor Ruboxistaurin has been in clinical trials to treat Diabetic Retinopathy. The hexosamine pathway intermediate glucosamine is toxic to mitochondria and promotes peroxide production. Benfotiamine, a B1 derivative, may inhibit AGEs, PKC, and hexosamine pathways. DM causes an imbalance of the pro-NGF/NGF ratio, promoting apoptosis. NGF eye drops show promise at treating DME by normalizing ratio. The BDNF ratio is also affected the same way. Constant supplementation of BDNF inhibits photoreceptor death, however routine injections are not effective.

Elevated TNF-α is seen in retinal tissue one week after DM onset, stimulating extrinsic apoptosis. Etanercept, TNF-$\alpha\beta$ inhibitor, appears to slow progression of DR. Hyperglycemia downregulates PI3K/Akt pathway, used for neuronal survival. Insulin promotes this pathway which protects from apoptosis, yet simultaneously promotes apoptosis. Muller cells and microglia are activated by hyperglycemia and release inflammatory mediators and cause glutamate excitotoxcity. Muller cell activation can be seen 1.5 months after DM onset, transient BBB breakdown within 6 weeks, and increased glial reactivity. Tau regulation is mediated by astrocytes. Abnormal tau causes astrocyte dysfunction and leads to neuron death.

Nitric Oxide gets inactivated by ROS forming peroyxnitrite and creating a neurotoxic environment. VEGF promotes neuron survival at low levels, but apoptosis by degradation of BDNF and GNDF at high levels. Elevated ROS promotes VEGF and inhibits its protective effects.

Conclusion

Several mechanisms for neurodegeneration preceding diabetic retinal vasculopathy have been described, both cellular and molecular. Many studies detail the potential for neurodegenerative pathway to lead to retinal vasculopathy. Continued research on which mechanisms predominate is necessary to develop effective treatments to prevent the onset of DR.

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1. Introduction

Background on Diabetic retinopathy

Diabetic retinopathy (DR) remains a widespread complication of diabetes mellitus (DM), affecting 34.6% of an estimated 415 million diabetic people globally. [1,2] DR has historically been understood to be a vascular condition because of the vascular abnormalities noted during the progression of the disease. In early stages of clinically appreciable DR, the presence of vascular abnormalities such as microaneurysms and dot/blot hemorrhages are seen across the retina and primarily in the macula.[3] The patient is most often visually asymptomatic at this time. This is deemed nonproliferative diabetic retinopathy (NPDR). Later stages of DR are distinguished by the presence of neovascularization, whether that be neovascularization of the angle (NVA), the iris (NVI), the disc (NVD), or elsewhere (NVE). This is diagnosed as proliferative diabetic retinopathy (PDR). Proliferation is often detected by the presence of leakage on fluorescein angiogram or the presence of a vitreous hemorrhage. In either stage of DR, increased vascular permeability can form diabetic macular edema which can cause the patient to experience visual impairment or distortion. When active proliferation is detected, intravitreal anti-vascular endothelial growth factor (anti-VEGF) treatment is initiated to control neovascularization. Intravitreal anti-VEGF treatment is also used to reduce macular edema if the edema is causing visual impairment. Treatment is performed at intervals typically between 4 and 8 weeks. There is no current treatment for NPDR or treatment to prevent the onset of the disease.

Previous research has extensively detailed the mechanisms by which chronic hyperglycemia leads to retinal vasculopathy, including the roles of endothelial cell dysregulation, decrease in retinal perfusion, and formation of acellular retinal capillaries, resulting in abnormal angiogenesis.[4] However, new research shows an increasing significance of neurodegeneration in the pathophysiology of DR. Mechanisms of neurodegeneration are distinct from vasculopathy. Imaging studies of diabetic eyes using optical coherence tomography (OCT) show that structural neurogenic changes are present prior to the onset of DR, leading researchers to believe neurodegeneration may precede vascular abnormalities.[5,6] Several studies have been done on diabetic patients exploring where this neurodegeneration is taking place and what common patterns can be identified. Araszkiewicz et al. compared OCT images of Type 1 Diabetes Mellitus (T1D) patients with DR, T1D patients without DR, and a control group.[7] Retinal thickness among T1D patients both with and without DR were significantly different than the control group,

specifically the retinal nerve fiber layer (RNFL) and ganglion cell layer (GCL). Therefore, apoptosis of retinal ganglion cells is believed to be one of the initial abnormalities in the diabetic retina, preceding vasculopathy.[8,9] Similarly, Tavares Ferreira et al. analyzed OCT images of diabetic patients without a diagnosis of diabetic retinopathy compared to a control group and found the diabetic patients had significant thinning of the photoreceptor layer.[10] Structural neurogenic changes affecting neural retina layers before the onset of diabetes-induced vascular abnormalities has similarly been observed in several other recent studies[5,11,12] To explain this phenomenon, it has previously been hypothesized that direct effects of hyperglycemia and hypoinsulinemia are responsible for neuronal degeneration.[10] Additionally, neurological degeneration of the retina in early DR has been observed as a potential separate phenotype to vascular DR, further indicating that the mechanisms by which neurons degrade in DR is distinct from vascular pathology.[13,14] One study in diabetes-induced mice found neurodegeneration progressed in mice that were genetically vasculoprotected.[15] This result emphasizes the theory that the initial retinal neuron damage due to diabetes is independent of vascular damage.[16]

In addition to finding mechanisms of neurodegeneration being independent of and preceding vasculopathy, recent studies have also thoroughly explored how retinal neurodegeneration may itself precipitate vascular damage over time.[17] The relationship between the retinal neurons and vasculature is a growing area of research, as this neurovascular unit (NVU) is what creates the blood retinal barrier (BRB). Disruption of the BRB is a hallmark of diabetic eye disease. [14] Previous articles have suggested that neuronal abnormalities of the retina occur in the primary stages of the disease, feeding into the breakdown of the blood-retina-barrier, inducing a toxic environment leading to vascular dysfunction.[8] One previous study showed focal areas of retinal ganglion thinning were associated with observed decrease in vascular density in the same locations.[3] Multiple studies detailing the relationships of signaling molecules within the retina in DR have shown that damage to neurons and glial cells result in VEGF production and subsequent microvascular damage.[18] Factors in neurodegeneration such as ROS, AGEs, and inflammatory factors have been shown to be factors in retinal-blood-barrier breakdown as well.[19]

Recent academic focus has been on the latest research regarding the pathophysiology of DR.[14] However, due the growing evidence pointing to neurodegeneration as the first sign of disease, it is necessary to detail the mechanisms of neurodegeneration in order to effectively target DR from the earliest signs of disease. Current treatment is only able to target abnormal angiogenesis at the end-stage of the disease. Furthermore, preventative measures focusing on good glycemic control have been shown to be ineffective in halting progression of neurodegeneration in DR, and the disease has been shown to progress in those with prediabetes as well.[20, 21, 22] Understanding the mechanisms by which neurodegeneration occurs in DR will allow therapeutic intervention to prevent the onset of vascular pathology, which remains the primary cause of vision loss in DR. This review article will explain the main cellular and molecular mechanisms of neuronal degeneration in DR Briefly, hyperglycemic conditions result in inflammatory increase, reactive oxygen generation, and glycation end products. These abnormal products then globally affect the retinal environment, starting cascades which degrade neuronal survival and function. A summary of this review is found in Figure 1.

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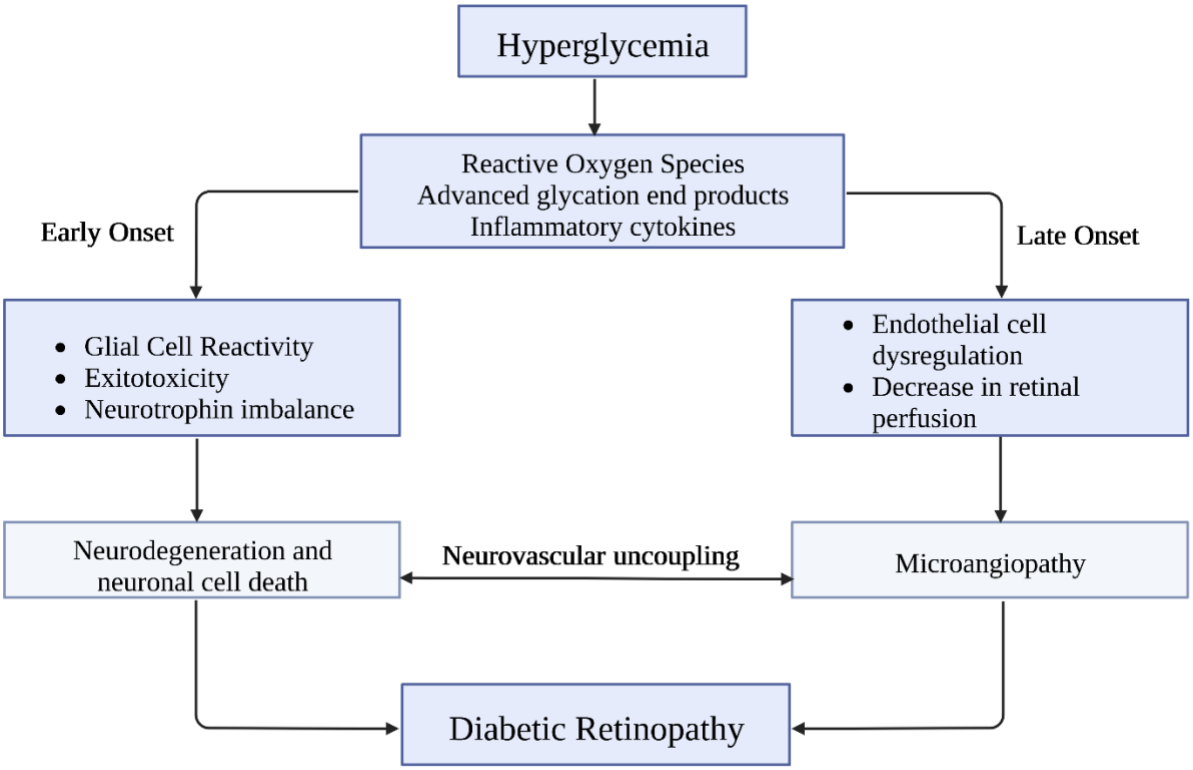

Figure 1. Summary schematic detailing mechanisms in the pathogenesis of diabetic retinopathy. The roles of neurodegeneration and microangiopathy are outlined.

2. Cellular and Molecular Mechanisms of Neuronal Degeneration

Multiple features of the retina predispose it to DR. To perform its physiologic function, the retina must transmit light with the least alteration possible. For this purpose, the retina contains a low density of blood vessels, unmyelinated axons, and few mitochondria to reduce disturbances in light transmission. However, these features also increase the metabolic burden of neuronal cells.[23] This inherent susceptibility encourages multiple mechanisms of neurodegeneration: oxidative stress and mitochondrial dysfunction, apoptotic signaling, glial reactivity, glutamate excitotoxicity, and neurotrophin imbalances.[20]

2.1 Molecular Mechanisms of Neuronal Degeneration

2.1.1 Reactive oxygen species and mitochondrial dysfunction

Reactive oxygen species (ROS) have been shown to be implicated in retinal neurodegeneration in the setting of diabetes in animal models and in humans.[24,25] Under physiologic conditions, the retina is a hypoxic environment, a situation that is exacerbated by the diabetes disease process.[26] Hyperglycemia-induced oxidative stress has previously been shown to

induce apoptosis in neuronal cells.[27,28] Visual impairment in early DR has been linked to effects of oxidative stress.[29] Hyperglycemia results in multiple pathways that end in the generation of ROS. The four main pathways are as follows: polyol pathway, hexosamine pathway, advanced glycation end products (AGE) pathway, and the protein kinase C (PKC) pathway. Although the production of ROS in diabetes by these mechanisms is not specific to neuronal cells, it has been shown that photoreceptor cells are a major producer of superoxide in DR due to their relatively higher number of mitochondria possessed, higher ATP consumption, and are the most vulnerable to oxidative stress.[30-32] It is crucial that the individual pathways of ROS production continue to be explored, as the effects of ROS generate much of the initial neurodegeneration.[26] Through understanding each pathway, therapeutic agents can be created to combat and prevent cell destruction through mechanisms like pathway modification before clinical signs of disease are present.

In the polyol pathway, glucose is converted into sorbitol and subsequently into fructose. This process requires usage of sorbitol dehydrogenase and aldose reductase, as well as their respective co-factors, nicotinamide adenine dinucleotide phosphate (NADPH) and nicotinamide adenine dinucleotide (NAD+).[30] In hyperglycemic conditions, the polyol pathway is more active, leading to a decrease in NADPH. NADPH also serves as a co-factor to regenerate glutathione, an antioxidant enzyme. Thus, in hyperglycemic conditions, tolerance to oxygen radicals is decreased. Fructose, the end-product of this pathway, may be oxidized further as well, however this is performed by sorbitol dehydrogenase (SDH), which is scarce in tissues like the retina. Because of this, retinal cells accumulate large amounts of sorbitol. Fructose and sorbitol are difficult to remove from the cell and thus increase osmotic pressure within the cell. This causes cellular swelling and rupture. Additionally, the increased production of NADH from NAD+ also promotes cellular edema and metabolic stress.[33] Being that aldose reductase converting glucose to sorbitol is the rate-limiting step and the direct cause of the accumulation of sorbitol in cells, it is a highly sought after target for potential therapeutic intervention.[34,35] At this time, aldose reductase inhibitors (ARIs) are not used for the treatment of DR, but the ARI Epalrestat is FDA approved for the treatment of diabetic neuropathy. This signals the potential for future ARIs that can treat early DR, before irreversible retinal changes take place.

In the hexosamine pathway, glucose is recruited to eventually produce glycosyl side-chains side-chains for modification of proteins and lipids.[30,33] In hyperglycemic conditions, this pathway is hyperactivated, leading to an increased production in glucosamine, an intermediate of the hexosamine pathway. Glucosamine is toxic to mitochondria and stimulates production of H_2O_2, further resulting in oxidative stress. Increased ROS within the cell may also perpetuate the hexosamine pathway by reducing the activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), causing glycolytic products to enter the hexosamine pathway. This shunting also induces the production of AGE products.

In the PKC pathway, diacylglycerol production, and thus PKC activation, is increased due to the increased activity of the glycolysis pathway. PKC promotes the activity of NADPH oxidase and induces the production of ROS by this mechanism.[30,32] NADPH oxidase is the main producer

of ROS in the cytoplasm and has been shown to occur even before ROS-induced mitochondrial dysfunction.[36] PKC inhibitors, like Ruboxostaurin, are potential therapeutics that could limit the ROS produced by this pathway.[37] However, there are no approved PKC inhibitors for the treatment of DR, at this time.

In the AGE pathway, hyperglycemia causes non-enzymatic glycation of macromolecules, indirectly increasing ROS production. Previous reports have extensively studied AGE in the context of vasculopathy, however AGEs have also been directly linked to neuronal death in DR.[33,38] Upregulation of receptors for advanced glycation end products (RAGE) in retinal ganglion cells has been observed in DR which may account for neurodegeneration, and AGE has also been directly linked to retinal neuronal degeneration in at least two previous studies.[38-40] AGEs cause dyslipidemia, resulting in further activation of the PKC pathway. This is especially prominent in all retinal cells due to its composition of polyunsaturated fatty acids.[24] Additionally, the interaction between AGEs and RAGE has been shown to activate NADPH oxidase and therefore upregulate the production of ROS.[20] In order to combat these destructive pathways, significant focus has been placed on vitamin supplementation.[30] Specifically, the hexosamine, PKC, and AGE pathways have all shown to be impeded after the administration of a vitamin B1 derivative, Benfotiamine. This is because Benfotiamine functions to activate transketolase, which then depletes fructose-6-phosphate and glyceraldehyde-3-phosphate. This ultimately inhibits all three of the previously stated pathways, and therefore may be a potential agent to inhibit ROS production.

The effects of ROS largely implicate mitochondrial damage. Previous studies have shown that mitochondrial DNA is the first to be damaged by ROS.[17]. In hyperglycemic conditions, the mitochondrial electron transport chain reaches its voltage limit, resulting in a backwards flow of electrons back to coenzyme Q and to oxygen, leading to the formation of oxide radicals.[25,30] A recent study on rat Müller cells showed that high glucose (HG) treatment induced ROS productions which then activated the extracellular signal reactive kinase (ERK) pathway.[41] This activation then promoted autophagy. In the setting of DM, where glucose levels are chronically elevated, this activation will promote Müller cell death. It has also been shown that oxidative stress from excess ROS produced by HG levels promotes calcium entry and, therefore, neurodegeneration.[36]

ROS also induce epigenetic modification. DNA methylation, histone methylation, and the oxidation of histone deacetylases have all been shown to occur following oxidative stress in retinal microvasculature.[30] In this context, histone modification has been closely tied to the inhibition of the body's defense against ROS.[42] ROS also appear to have significant effects on the actions of sirtulins, which are proteins that modulate processes like cellular metabolism through the action of deacetylation. SIRT1 and SIRT6, both of which have been shown to be inhibited in DR, function to inhibit inflammation from NF-kB activation. A study performed on SIRT6 knockout mice resulted in retinal neurons with increased apoptosis in the absence of vasculopathy.[43] This experiment demonstrates the potential importance of the relationship between ROS and epigenetic changes in retinal neurons, as it could be a major contributor to the initial neurodegeneration present in DR.

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2.1.2 Neurotrophic factors and imbalances in neuroprotective factors

Neurotrophic factors are biomolecules (short peptides) that are essential in neuronal cell survival, outgrowth, and differentiation, and belong to a larger class of growth factors.[44-46] Neurotrophin levels must be maintained throughout life for the continued survival of neurons. All neurotrophins are synthesized first in immature forms and are later modified by proteases to their mature form. Mature neurotrophins interact with a tyrosine kinase tropomyosin (Trk) receptor which eventually signals for cell survival and proliferation. Pro-neurotrophins, rather, can interact with $p75^{NTR}$, a tumor necrosis factor (TNF) receptor. Along with the transmembrane receptor sortilin, this will downstream signal for cell death. In healthy tissues, the ratio of pro-neurotrophins to neurotrophins is maintained at an equilibrium which allows for adequate survival of neural cells. During times of acute injury, neurotrophins are secreted and promote neural cell regeneration. However, this inflammation also causes secretion of pro-neurotrophins, which can lead to further neuronal apoptosis. Chronic inflammation, therefore, can have detrimental effects on neural cell tissues.

In the retina, Müller cells produce neurotrophic factors and growth factors necessary for neuron maintenance including brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), basic fibroblast growth factor, insulin-like growth factor, nerve growth factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4), glial cell line-derived neurotrophic factor (GDNF), leukemia inhibitory factor, and pigment epithelial-derived factor.[47] Of these, NGF is the most studied. Previous studies have identified a significant increase in pro-NGF levels and decrease in NGF levels in diabetic retinas, as well as in isolated retinal Müller cells after being treated with high glucose.[46] Elevated pro-NGF levels in the retina have been found to upregulate $p75^{NTR}$ expression, as well as inflammatory molecules like IL-1β and NF-kB in Müller cells therefore causing inflammation and photoreceptor death. Accumulation of pro-NGF has also been associated with suppression of TrkA, the receptor for NGF. A recent study in mice shows that when pro-NGF is overexpressed, there is notable neurodegeneration resulting in cognitive impairment. It is important to understand that the changes in pro-NGF/NGF levels and the resulting inflammation are identifiable before noticeable retinopathy, which points to this mechanism likely being a major contributor to the initial neurodegeneration seen in the diabetic patients without vasculopathy.

Restoring the pro-NGF/NGF ratio and inhibiting this cell death pathway offers the potential to prevent the initial neurodegeneration that precedes vasculopathy. In 2022, Fico *et al.* studied the effect of NGF administration by eye drops on streptozotocin-induced diabetic mice.[48] It was found that diabetic mice being administered NGF eye drops saw significantly less neurodegeneration as compared to the diabetic mice who did not receive the NGF eye drops. Far less apoptotic activity was seen in the treatment group, showing that supplementation of NGF has the potential to restore balance to the pro-NGF/NGF ratio and reverse the downregulation of the TrkA receptor. It is important to note, however, that NGF administration has been shown to promote angiogenesis. This would be harmful in patients with more severe DR. Therefore, more

research needs to be done on the potential this drug has for reversing or delaying the onset of DR. These results point toward the understanding that DR encourages neurotrophic factor imbalances and that levels may change as DR progresses, diminishing their neuroprotective effects.

BDNF is another neurotrophin produced by neurons and glial cells and is also pivotal in the development and function of neurons, as well as synaptic plasticity and synaptic function.[49,50] It has been demonstrated that diabetic patients have a low BDNF compared to controls, and is inversely related to blood glucose levels and insulin resistance.[51] Like pro-NGF, pro-BDNF stimulates apoptosis of neuronal cells, potentially by triggering caspase-dependent pathways.[51,52] Ola *et al.* found that BDNF is reduced in diabetic retinas and it is associated with neurodegeneration early in DR.[27] El-Asrar *et al.* found NT-3 and NT-4 to be increased in vitreous humor while NGF and BNDF were undetectable.[53] To test the potential therapeutic effect of BDNF, Okoye *et al.* studied transgenic mice with doxycycline-inducible BDNF expression and found that constant supplementation of BDNF is protective against photoreceptor death from oxidative stress.[54] This result was compared to older studies that showed no notable protective effects from regular intravitreal BDNF injections.[55] From these experiments, it can be inferred that BDNF treatment on intervals is insufficient to illicit a protective response.[54,55] More research needs to be done to explore the options of a sustained release preparation or the incorporation of BDNF in a combination of other neuroprotective agents.

Other prominent neurotrophins found in the human retina are NT-3 and NT-4. Boss *et al.* studied the neurotrophin levels in human retinas and found elevated NT-3 and NT-4 levels, as well as elevated levels of NGF, BDNF, CNTF, and GDNF.[56] At this time, neither NT-3 nor NT-4 have been tested as potential therapeutic agents. More research needs to be done to explore the role these neurotrophins play in the pathogenesis of DR.

Pigment epithelial-derived growth factor (PEDF) is a glycoprotein present throughout the body which has been shown to have antioxidant and anti-inflammatory properties.[57] In a diabetic milieu, this role is especially important to protect cells like retinal pericytes from ROS, AGEs, and a decreased anti-apoptotic to pro-apoptotic enzyme ratio like Bcl2/Bax. PEDF has also showed to be protective of retinal neurons against glutamate excitotoxicity and oxidative stress in DR.[23,59] PEDF has been found to be decreased in the vitreous and retina of those with uncontrolled diabetes.[58] This connection between decreased PEDF neuroprotection and the initial neurodegeneration involved in DR opens the potential for the use of PEDF to protect against or reverse early signs of disease.[59,60] Continued research is needed to determine the potential PEDF has as a therapeutic agent against DR.

2.2 Cellular Mechanisms of Neuronal Degeneration

2.2.1 Caspases and Apoptotic signaling

Caspases are cellular enzymes responsible for initiating programmed cell death, as well as promoting inflammation.[61,62] A significant amount of cellular research in DR is dedicated to

understanding the involvement of caspases in the involved apoptotic pathways, as caspase mediate most mechanisms of apoptosis. Apoptosis can be performed in multiple different pathways. The intrinsic pathway begins with a triggering event like DNA damage that causes pro-apoptotic mediators like BAX and BAD to permeate the mitochondrial external membrane. This allows for the leakage of cytochrome c from mitochondria to then interact with Afap-1 to form an apoptosome. This apoptosome activates caspase-9 which then activates caspase-3, -6 and -7. Caspase-3, -6, and -7 are a common denominator for apoptotic pathways and are called executioner caspases because they facilitate the destruction of the cell. The extrinsic pathway begins with an apoptotic signal originating from outside the cell, like Fas ligand binding or TNFα interacting with the TNF receptor. This causes the activation of caspase-8 and caspase-10. These activated enzymes then activate the executioner caspases to complete the apoptotic pathway. In chronic disease states like DM, these pathways can because unbalanced, leading to pathological cell death.[62] In the case of neuroretinal cells in early DR, it is believed this is vision-impairing damage.[63]

Current evidence shows that neuronal apoptosis in DR occurs via caspase-dependent and caspase-independent mechanisms.[32,64,65] Electrophysical examination has found that photoreceptors cells and retinal ganglion cells are the most affected by apoptosis.[64] El-Asrar *et al.* analyzed several postmortem diabetic retinas and found increased expression of caspase 3, Fas, and Bax in retinal ganglion cells.[66]. It was also found that the diabetic retinas showed *de novo* expression of BAD, whereas the nondiabetic retinas did not. Therefore, it is presumed that hyperglycemia itself appears to directly induce increased expression of BAD.[67] In a study on rats, it was found that two months after the onset of DM, there was no presence of caspase-3 in the retina.[68] However, after 14 months, caspase-3 activity was present, as was histopathology of DR. In the diabetic rats that were also treated with an antioxidant mixture, the caspase-3 level remained absent. These results suggest it is the oxidative stress secondary to hyperglycemia over an extended period which activates caspase-dependent apoptosis of retinal pericytes.

In the setting of diabetes, there is an association between the chronic inflammation due to hyperglycemia and glutamate excitotoxicity in neural cells that leads to neurodegeneration throughout the nervous system, including the neuroretina.[32] In healthy physiology, neurons can use glutamate as a neurotransmitter. When glutamate is transported into the synapse, it can be taken up by surrounding cells like astrocytes to then be recycled using Na+/K+-ATPase. Excitotoxicity can occur when these levels of glutamate released from neurons becomes extremely high. Excitotoxicity triggers neuron apoptosis. In the glutamate excitotoxicity present in DR, mitochondrial apoptotic pathways have been found to dominate. Mitochondria have been found to induce caspase-independent cell death, instead functioning by releasing apoptosis-inducing factor (AIF) and endonuclease G.[64,69] In diabetic patients, photoreceptors and ganglion cells especially have been found to have increased expression of AIF and cytochrome *c*. AIF is normally present in the mitochondrial membrane but can be translocated to the nucleus in the presence of cytotoxic stimuli. There it induces DNA fragmentation. This mechanism is does not require caspases, and was seen to cause retinal neuronal apoptosis without a concurrent rise in the effector caspase-3.[64]

One major pathway in which diabetic retinal ganglion cells become apoptotic is via the extrinsic lethal ligand signaling pathway.[70] In this pathway, a cell like a retinal ganglion cell will present a transmembrane death receptor on the cell membrane with a "death domain" on the cytosolic side. Examples of death receptors present in retinal ganglion cells include CD69 and TNF-α receptor 1. When a cell, like a T lymphocyte, binds Fas ligand (FasL) or a soluble molecule like TNF-α bind to their respective death receptor on the cell, the "death domain" recruits a collection of proteins called the "death-inducing signal complex."[71] This complex activates caspase-8 which then allows for the intrinsic apoptotic pathway to complete the process. This apoptotic mechanism has also been demonstrated to occur in diabetic retinal neurons, with subsequent activation of caspase-3.[72] Given the presence of several anti-inflammatory drugs already approved for autoimmune conditions, inhibition of these apoptotic pathways continues to be an area of particular focus.[73] Most of the current research has focused on the use of drugs like Etanercept, a TNF-α/β inhibitor fusion protein, to slow the progression of DR. However, it has been shown that the levels of TNF-α in the retina are elevated even one week after the onset of diabetes. Coupled with the fact that retinal ganglion cells are known to have death receptors for TNF-α, it is believed immunomodulatory drugs like etanercept may be promising interventions to prevent the early neurodegeneration seen in DR.

Anti-apoptotic factors have also been found to be decreased in retinal neurons in DR.[69] PI3K/Akt kinase pathway, a survival signal for neurons, is found to be decreased in diabetic rat retinas and contributes to neuronal cell death. Experimental studies in human neurons have produced the same result. A study using retinal neural model cells showed that the administration of insulin to retinal cells in a hyperglycemic environment showed a markedly reduced number of cells undergoing apoptosis compared to those without insulin administration.[74] This effect occurs because of the downstream effects of insulin binding to the insulin receptor, which is plentiful in the retina and can be specifically found on retinal neurons. This causes the activation of the PI3-kinase/Akt pathway that phosphorylates caspase-9, and therefore inhibits the activation of the executioner caspase-3. These results point to the potential for insulin to function as a potential therapeutic to prevent neurodegeneration, however evidence shows that insulin administration at high amounts may activate separate pro-apoptotic pathways.[62,74] Therefore, more research is necessary to identify techniques to circumvent this issue.

2.2.2 Glial reactivity and glutamate excitotoxicity

Glial cells are non-neuronal cells tasked to support surrounding neurons and maintain extracellular homeostasis.[75] Within the retina, these cells are responsible for neuroprotection, angiogenesis, metabolism, inflammatory responses within the retina, synaptic pruning, and development.[23,75] There are three forms present within the human retina: Müller cells, astrocytes, and microglia. Müller cells are the most predominant glial cell in the retina and are found in all layers. These cells ensheathe axons within the retina and form the blood-retina-barrier. Under physiologic conditions, Müller cells regulate neuronal activity by controlling neuroactive substances, potassium, and ATP in the ECM, as well as by secreting neurotrophic factors.[76]

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In response to conditions in hyperglycemia, Müller cells become activated, and contribute to neurodegeneration via glutamate toxicity, neurotrophin imbalance, cytokine release, and dysfunction of the neurovascular unit (NVU).[77] Neurotrophin imbalance and NVU dysfunction are discussed in previous sections. Markers for Müller cell activation such as glial fibrillary acidic protein (GFAP) have been observed both in animal and human diabetic eyes. When Müller cells are activated, they begin to release inflammatory cytokines like IL-1β, IL-6, and TNF-α. They will also secrete growth factors like VEGF and PEGF. This process is also referred to as reactive gliosis. Müller cell gliosis affects every cell type in the retina, and overall contributes to both neuronal and vascular degradation by establishing an inflammatory environment within the retina.[49,77] Müller cells also directly cause neurodegeneration by glutamate excitotoxicity. Under normal physiologic conditions, Müller cells are responsible for regulating neurotransmitters in the extracellular space. They take up excess glutamate and convert it to glutamine or alpha-ketoglutarate.[20,27] Glutamate is the main excitatory neurotransmitter in the retina and is used in transmission from photoreceptors to bipolar cells and bipolar cells to retinal ganglion cells, which includes more than 90% of synapses in the retina.[49,78] However, in hyperglycemic conditions, uptake of glutamate by Müller cells is impaired, and glutamate conversion to glutamine and alpha-ketoglutarate is reduced, perhaps due to increased ROS within Müller cells in hyperglycemic states.[20,78] The resulting excess extracellular glutamate causes NMDA overactivation in surrounding neurons.[49] Calcium influx has also been seen to potentiate glutamate excitotoxicity in postsynaptic neurons by several mechanisms.[78-58] Mitochondria tend to sequester calcium in situations of glutamate toxicity, leading to metabolic acidosis and ROS generation. Calcium influx also induces activation of caspases from the mitochondria, leading to apoptosis.[78] Calcium influx has been shown to be uncontrolled in postsynaptic neurons in glutamate excitotoxicity.

Understanding the detrimental effects of Müller cell activation has been of particular focus in the fight against DR related retinal neurodegeneration, because of the key relationship between Müller cells and retinal neurons.[78] It is important to consider, however, that Müller cell activation is an attempt to counter diabetic insult to neurons. Should Müller cell gliosis be a potential therapeutic agent to counter this cellular response, the possible consequences of inhibiting this inflammatory protection must be considered.

Astrocytes are also present in the retina to a lesser degree.[75] Their functions largely overlap with those of Müller cells: they ensheathe retinal neurons, regulate the extracellular environment, constitute the BRB, and function to support neuron survival. However, astrocytes can migrate independently of vessels are provide layer-specific modulation in the retina. Despite this ability to migrate freely, most astrocytes reside in areas with thicker nerve fibers. Meanwhile, in areas like the ora seratta and fovea, where the nerve fiber layer is extremely thin, there is a notable absence of astrocytes. This finding highlights the significant relationship astrocytes and RGCs. By contrast, Müller cells are present at all layers and must respond to insults in a layer-nonspecific manner. Astrocytes have been observed to proliferate, migrate, and secrete pro-inflammatory markers like IL-6 and TNF-α in the setting of DR.[79] Studies both in vitro and in vivo have also demonstrated astrocyte apoptosis in the setting of hyperglycemia. Hyperglycemic conditions have also shown to stimulate reactions from astrocytes like cytokine release, oxidative stress, and stimulated NF-kB.[80]

In both normal and pathological conditions causing retinal neovascularization, astrocytes are known to be the primary cell type producing VEGF. Despite not being the most abundant glial cells in the retina, astrocytes are believed to play a critical role in ion homeostasis and therefore are of increasing focus around cellular contributions to DR. A new topic of increasing interest is the potential effects of tau on retinal astrocytes and its implications in DR-related neurodegeneration.[81] Tau is a protein present in neurons that functions to stabilize microtubules. In Alzheimer's disease, abnormal tau has been shown to contribute to axonal degeneration.[82] This is believed to occur due to neurons secreting abnormal tau which is then taken up by nearby astrocytes. The absorbed tau then interferes with several cell processes within the astrocyte that ultimately hinders neurotransmission. This process has yet to be researched in retinal cells, but it is believed to be a potential mechanism in DR-related neurodegeneration.

Microglia are also present within the retina.[23,75] These cells act as macrophages within the retina in addition to sharing responsibilities with astrocytes and Müller cells. Their motility is fueled by ATP in the extracellular space as released by Müller cells, and Müller cells have been seen to potentiate the activation of microglia cells. Microglia can also act in conjunction with astrocytes to modify synaptic contacts as well as increase inflammatory responses.

Hyperglycemia leads to the production of ROS, which activates microglia.[83] Microglia have also been observed to proliferate in response to hyperglycemia *in vitro.* Microglia have been shown to be activated by hyperglycemia, ischemia, hypoxia, and dyslipidemia but the exact mechanism is not yet fully understood. It is known that in response to ROS, microglia produce cytokines, TNF-α, pro-inflammatory interleukins, NFκB, and TLR-2 and TLR-4.[84] Retinal neuronal death has been found to be increased by TNF-alpha mediated caspase 3 activation. These cells may also be activated by AGE-RAGE connections. When activated, microglia also develop an amoeboid appearance from their quiescent ramified forms.[85] In this activated form, microglia migrate to the plexiform layers early in diabetic retinopathy.[23,30] There, the presence of inflammatory factors as well as the production of glutamate, ROS, caspase-3, and nitrous oxide is increased. These factors contribute both to neurodegeneration and to the breakdown of the BRB. Like astrocytes, abnormal tau activity has also been associated with microglia activation and, ultimately, neurodegeneration. Studies on microglial activation have identified microglial secretion of IL-1α and IL-1β to hyperphosphorylate tau and downregulate synaptophysin.[86] These effects have been shown to cause synapse loss and, later, neuron apoptosis. This pathway has only been researched in the context of Alzheimer's disease at this time, however it is believed it could play a role in DR-related neurodegeneration.[82]

Animal studies have shown that glial reactivity is linked to increased neurodegeneration in DR.[4] Pannicke *et al.* found that within 1.5 months of diabetes induction, Müller cells are activated, and transient BRB breakdown occurs within 6 weeks of diabetes induction in rats.[87] Neurodegeneration is detected within 6 months of diabetes onset and is associated with increased glial reactivity.[4] Because glial cells are the major regulators of the extracellular retinal environment, they are a very important consideration when it comes to understanding the pathogenesis of neurodegeneration and the potential pharmacological interventions for neuronal

protection.[82] Research is currently being done exploring the potential for glial cell activation inhibitors and neuro-protective compounds to counter and prevent this disease process.

3. Vascular contributions to neurodegeneration via the neurovascular unit

Examination of neurodegeneration in DR and its mechanisms cannot be fully separated from its vascular counterpart. As more evidence has come to light, DR is now considered a neurovascular disease, as appreciated through the lens of the "neurovascular unit" (NVU).[4] The NVU describes system in which neurons, vascular cells, and supporting glial cells function together to maintain neuronal homeostasis and appropriately functioning vasculature. Vascular abnormalities have been studied extensively in late DR; however, some findings have also suggested that vascular injury may be found early in DR, alongside neurodegeneration.[74,88] Some of the earliest noticeable signs of microvasculopathy in DR are microaneurysms. However, vascular damage may be present before it is evident upon fundus examination in the form of acellular capillaries. Animal models have helped researchers better understand the timeline of microscopic retinal changes after the onset of diabetes. In a study on rats, it was found that Muller cells were activated at about 1.5 months after onset of DM.[4] Apoptosis of pericytes began at around 4 months after DM onset. Then, at 6 months, acellular capillaries began to form. Meanwhile, electroretinography on the rats showed neuronal dysfunction starting at about 6 weeks after DM onset. This suggests a potential pathway in which neurodegeneration may initiate vascular abnormalities: Neuronal stress from hyperglycemia activates glial cells which then create inflammation that gives rise to vascular injury.[89, 90]

Neurotrophic factors that have been previously discussed regarding their effects on neurons and glial cells have also been studied on their effects on retinal vasculature. PEDF, a glycoprotein previously described as being neuroprotective, has been shown to also decrease neovascularization in the setting on DR.[91] Similarly, microglial cell reactivity in early DR has not only been linked to neurodegeneration, but also the formation of acellular capillaries.[92] Additionally, proNGF has been found to induce the development of acellular capillaries, showing that growth factor imbalances may result in both neuronal and vascular damage, and initiate a feedforward mechanism resulting in the progression of DR.[93] The production of damaging substances like ROS by one component of the NVU can have direct effects on neighboring cells. For example, photoreceptors may contribute to vascular damage in DR as they generate ROS and inflammatory proteins.[93] Because of the entanglement of neurodegeneration and vascular injury, it is necessary to question whether there are microvascular contributions to neurodegeneration early in the disease.

3.1 Nitric oxide production

Under physiologic conditions, the retina is a hypoxic environment.[93] Oxygen consumption changes with the amount of light shining on the retina, with oxygen consumption being greater in

dark environments. However, blood vessels do not respond directly to light, and thus are reliant on signals from neurons and glial cells to mediate neurovascular coupling.[88] For this NO is crucial for vascular control; each cell in the retina is able to produce NO. However, ROS produced in DR renders NO less available through the interaction of free radicals with NO and the production of peroxynitrate.[94] This is evidenced by the increased serum levels of nitrous oxide breakdown products in diabetic patients. Increased levels of oxidative products of NO result in abnormal vascular relaxation and contributes to a neurotoxic environment.

NO is synthesized by nitric oxide synthase (NOS).[95] In the retina, NOS is present in three different isoforms: inducible-NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). Research using diabetic NOS knockout mice has allowed us a better understanding of what role each NOS isotype plays in the pathogenesis of DR by comparing the diabetic NOS knockout group to non-diabetic knockout mice, diabetic wild type mice, and non-diabetic wild type mice.[95] In researching the iNOS isotype, it was found that diabetic $iNOS^{-/-}$ mice, compared to wild type diabetic mice, did not have many of the retinal characteristics of DR including acellular capillaries, elevated NO levels, and pericyte apoptosis. However, both groups showed similar GCL thinning. In diabetic $eNOS^{-/-}$ mice, it was found that these mice, compared to wild type diabetic mice, developed earlier onset retinopathy, more aggressive retinal vasculopathy, and sustained gliosis. NO levels in the diabetic $eNOS^{-/-}$ mice were also much higher than any other group, which is understood to create a neurotoxic environment for retinal neurons. By studying the roles of each NOS isotype, it can be easier to understand risk factors for severe DR and potential methods of treatment.

3.2 VEGF activity

Müller cells, as mentioned previously, are responsible for maintaining the neuro-vascular relationship.[96] One prominent role Müller cells play in neurovascular coupling is the production of VEGF. VEGF is widely known for its angiogenic effect. This activity is what is being inhibited by intravitreal anti-VEGF therapy in the case of retinal diseases like neovascular AMD or PDR. However, VEGF has a dose dependent neurotrophic effect on neurons.[75] At low concentrations, VEGF has shown to have a neuroprotective effect on retinal neurons, but at high concentrations it has shown to be damaging to retinal neurons. In the setting of diabetes, VEGF is being maintained at high levels, therefore causing neuronal damage. High levels of VEGF are believed to promote degradation of neuroprotective BDNF and GNDF.[96] It has also been shown that VEGF triggers vascular leakage, neovascularization, and vascular lesions. However, neurodegeneration has also been shown to increase when VEGF signaling is disrupted. VEGF signaling is seen as crucial to Müller glial cell viability, which may help neuroprotection in DR. These conflicting effects makes it necessary to weigh the pros and cons of long-term anti-VEGF and continue to seek improved interventions. While it is important to control the vascular damage VEGF causes to prevent permanent vision loss, many patients on long-term anti-VEGF have demonstrated a slow decline in visual acuity over time. This could be for many different reasons, and one theory is anti-VEGF injections compromising retinal integrity over time. While slow decline in vision over time is not

nearly as devastating as the vision loss without treatment, this possible adverse outcome necessitates the continued research into safer long-term treatments.

VEGF is known to be upregulated in settings of increased ROS.[97] As it has been established, chronic hyperglycemia in diabetes facilitates an elevated baseline ROS level. ROS are also believed to counter the protective effects VEGF has in the retina while promoting its damaging properties. Given retinal neurons are known to be negatively affected by high levels of VEGF, these compounding effects due to ROS make the damage especially destructive.

Conclusions

DR is a common complication of diabetes in which every cell type in the retina is affected or damaged, resulting in irreversible vision loss. Although DR was classically considered a vascular disease, evidence now supports DR as a neurovascular disease, in which neuronal damage is present much earlier than vascular pathology or clinically observable abnormalities. Viewing the physiology of the retina through the lens of the NVU has been pivotal to understanding the complex pathology of diseases like DR. Recognizing that neither retinal neurons nor the retinal vasculature reside in a vacuum, but rather in a dynamic environment with each other has allowed our understanding of DR to expand greatly. The mechanisms by which neurons are damaged in early DR are linked to the effects of reactive oxygen species, apoptotic signaling by caspase-dependent, caspase-independent, and Fas/FasL pathways and glial cell reactivity resulting in excitotoxicity, neurotrophin imbalance, and breakdown of neurovascular coupling. Although many features of neurodegeneration in early DR have been uncovered, research must continue to elucidate gaps in areas like understanding the effects of neurotrophins on the diabetic retina and the exact mechanisms by which ROS encourage neuronal apoptosis. These relationships are described in Fig 1.

These mechanisms are crucial to prevent progression of DR and vision loss. Currently treatment for DR solely addresses vascular symptoms but are ineffective to halt the disease or prevent its onset. Extensive research is currently being done to find new treatment options for DR, especially in its early stages. Because neurodegeneration is one of the first signs of disease, it has been of particular interest to researchers. Because of the direct effect retinal neurons have on the vasculature, it is possible that inhibiting neurodegeneration can prevent the onset of DR. Therefore, understanding the mechanism of neurodegeneration is a continued area of interest. As discussed in this paper, strict glycemic control in early stages of DR is not sufficient to prevent progression of neuronal apoptosis. Thus, understanding mechanisms of neurodegeneration in DR will guide future avenues of treatment development.

Acknowledgements

Cellular and Molecular Mechanisms of Neurodegeneration in Early-Stage Diabetic Retinopathy
A. Callan[1] (presenter), S. Jha[1], L. Valdez[1], A. Tsin[1]
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Supported by NIH grants (EY03351; CA278054; DA056203).)

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