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Connexin43 hemichannels: A potential drug target for the treatment of diabetic retinopathy

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Diabetic retinopathy (DR) is a chronic vascular disease of the retina that causes vision loss in patients with type 1 and type 2 diabetes, and is associated with vascular dysfunction and occlusion, retinal oedema, haemorrhage and inadequate growth of new blood vessels. Current DR therapies primarily target downstream, later-stage vascular defects with a significant proportion of diabetic macular oedema patients being non-responders. Moreover, other evidence suggests that prolonged use of therapies targeting vascular endothelial growth factor (VEGF) might be associated with increased onset of geographic atrophy and retinal ganglion cell death. It is therefore highly desirable to prevent the onset of DR or arrest its progression at a stage preceding the appearance of more-advanced pathology by targeting upstream disease mechanisms. Connexin43 hemichannels play a part in the pathogenesis of chronic inflammatory diseases, including inflammasome pathway activation; and hemichannel block has been shown to alleviate vascular leak and inflammation. This review discusses the inflammatory changes occurring in DR as well as current therapies and their limitations. It then focuses on the role of connexin43 in DR, providing evidence for the utility of connexin43 hemichannel blockers as novel therapeutics for DR treatment.

Introduction

Diabetic retinopathy (DR), the most common microvascular complication of diabetes, is an ocular degenerative disease that can lead to vision loss. DR is generally classified into three categories: nonproliferative or early-stage DR (NPDR), proliferative or late-stage DR (PDR) and diabetic macular oedema (DME). NPDR is characterised by the presence of micro-aneurysms, cotton-wool-like spots caused by infarcts in the retinal nerve fibre layer (NFL), and hard exudates [1]. As NPDR progresses, damage to the microvascular network of the retina becomes extensive, thereby increasing the risk of PDR, a condition induced by retinal ischaemia that results from a loss of vascular integrity which in turn leads to poor retinal perfusion [1]. Retinal ischaemia also induces neovascularisation, a pathological angiogenic process that worsens the disease by increasing leakage of plasma and debris into the retina. DME occurs when fluid accumulates in the macula, the light-sensitive area of the retina.

The current mainstay treatment for DR involves intravitreal injection of anti-vascular endothelial growth factor (anti-VEGF) agents to limit neovascularisation. However, anti-VEGF drugs have the potential to target not only damaged but also healthy blood vessels required to maintain retinal function or restore perfusion to ischaemic tissue. Indeed, there is evidence in recent studies that long-term use of anti-VEGF agents can result in geographic atrophy and loss of retinal neurons [2,3]. Furthermore, ~50% of DME patients do not respond to anti-VEGF drugs [4], which only affect late-stage pathologies in DR and do not target upstream DR pathways. As a result, alternative treatment avenues

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are being actively sought in an attempt to target the disease at its earlier stages.

Connexin43 hemichannel blockers have been found to be efficacious in various acute and chronic inflammatory disease models with a vascular disruption component, such as cerebral stroke [5] and spinal cord injury [6]. Recent studies have also confirmed these blockers to be effective in reducing inflammation and vessel leak in rodent models of retinal diseases such as retinal ischaemia [7], age-related macular degeneration (AMD) [8] and DR [9]. Connexin43 hemichannel blockers can prevent activation of the inflammasome pathway of the innate immune system, which is directly linked to activation of inflammatory processes through the release of proinflammatory cytokines and the loss of vascular integrity [10]. In this manner, connexin43 hemichannel blockers work upstream of anti-VEGF drugs to reduce inflammasome activation and inflammation in the first instance with the potential to prevent neovascularisation and, ultimately, DME. This review discusses the inflammatory processes occurring in DR, as well as current treatment strategies and their limitations. It concludes with evidence in support of the use of connexin43 hemichannel blockers as a potential therapy for the treatment of DR.

Key inflammatory changes in diabetic retinopathy

Despite the fact that hyperglycaemia is considered the main instigator of diabetes-induced damage to the retina, recent preclinical and clinical studies support the idea that DR is also associated with persistent and chronic inflammation characterised by increased levels of inflammatory mediators, leukocyte adhesion and activation, as well as reactive oxygen species (ROS) formation [11]. Therefore, it is likely that hyperglycaemia and inflammation are factors that together stimulate detrimental metabolic and functional pathways damaging the retina and resulting in vision loss [12]. The finding that anti-inflammatory therapies such as corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs) can significantly slow down the progression of the disease in a large proportion of the population [13] supports this hypothesis. However, such therapies have been unable to halt the disease altogether, possibly because they often target only one pathway and do not account for complex multi-inflammatory processes that probably act together in the disease. The following section briefly describes the inflammatory mechanisms underlying the pathogenesis and pathophysiology of DR.

Vascular endothelial growth factor

Several clinical and nonclinical studies provide evidence that elevated VEGF levels are associated with various pathological features of DR [14,15]. VEGF, an endothelial-cell-specific growth factor, is thought to increase endothelial cell proliferation, vessel permeability and blood flow in retinal blood vessels [16]. Boulton *et al*. were the first to show that VEGF levels are low in normal human retinas but increased in diabetic eyes [14]. Their study found that VEGF was localised to endothelial cells and perivascular regions, correlating with vascular lesions, and that VEGF levels increased with increasing disease severity [14]. These findings mirror previous research carried out in streptozotocin (STZ)-induced diabetic rats by Murata and colleagues, where increased VEGF expression and blood-retinal barrier (BRB) breakdown correlated with longer diabetes duration [17]. They also studied VEGF expression patterns within different retinal cell types and found that VEGF levels were increased in ganglion cells, glial cells with cell processes close to retinal vessels, pericytes and the retinal pigment epithelium (RPE) in STZ-induced diabetic rats compared with controls. VEGF localisation on cells associated with blood vessels and the understanding that VEGF causes endothelial cell proliferation suggest that VEGF worsens DR by stimulating BRB breakdown and neovascularisation [18,19]. Although inhibition of VEGF suppresses retinal neovascularisation *in vivo,* the fact that VEGF is also required for the maintenance of the vasculature means complete inhibition will not only affect abnormal but also normal blood vessels [20,21]. These studies show that, although VEGF acts on endothelial cells to mediate endothelial hyperpermeability and neovascularisation, which in turn contributes to deleterious mechanisms associated with DR, the physiological roles of VEGF are also important. Therefore, complete removal of VEGF might be associated with increased side effects.

Inflammatory cytokines

One of the first studies to evaluate inflammatory cytokines in DR was conducted by Powell and Field [22]. It showed that diabetic patients taking the anti-inflammatory drug salicylic acid had a lower incidence of DR. These findings triggered extensive research into the role of inflammation in DR. Krady *et al*. used an STZ-induced diabetic rat model to show that microglial activation is an early and necessary event in DR and that hyperglycaemia can directly increase the mRNA levels of two important proinflammatory cytokines: interleukin (IL)-1β and tumour necrosis factor (TNF)-α, which activate various inflammatory pathways [23]. Furthermore, minocycline, an antibiotic with anti-inflammatory properties, significantly decreased TNF-α-induced cyclooxygenase (COX)-2 expression in microglia, retinal neuronal cell death triggered by activated microglia and diabetes-related apoptosis in the retina of STZ-induced diabetic rats [23]. This study showed that hyperglycaemia, in conjunction with inflammatory mediators, could stimulate disruptive pathways that lead to DR. These findings were supported by Brucklacher *et al*. who showed increased expression levels of several proinflammatory cytokines in the retina of STZ diabetic rats [24]. Demircan *et al*. found significantly higher levels of IL-1β and TNF-α in the vitreous humor and serum of patients with PDR compared with controls [25]. In addition to IL-1β and TNF-α, several studies have suggested that monocyte chemoattractant protein (MCP)-1, a monocyte recruitment protein, is also increased in DR [26,27]. Unlike IL-1β and TNF-α, which are directly linked to hyperglycaemia, MCP-1 expression can be stimulated by VEGF [28,29]. Taken together, these studies highlight the significant role of inflammatory cytokines and chemokines in DR pathology, especially regarding inflammation and the inflammasome pathway.

Inflammasome pathway activation

The inflammasome was initially described as a large molecular entity that activates caspases and induces activation of IL-1β by cleavage of its inactive form: pro-IL-1β [30]. It is now well known that the inflammasome is a complex made up of nucleotide-binding domain and leucine-rich repeat-containing (NLR) protein-3 (NLRP3), apoptosis-associated speck-like proteins containing a caspase recruitment domain (ASC) and pro-caspase 1 [31].

The inflammasome can be induced by a plethora of different molecules that trigger complexation of the inflammasome components and a conformational change that converts pro-caspase 1 to caspase 1, which in turn cleaves and activates the inflammatory molecules IL-1β and IL-18. However, the inflammasome is associated with increased expression of several other inflammatory cytokines including TNF-α, IL-6, IL-8, MCP-1 and, of particular significance in retinal disease, VEGF [10]. To date, five NLRs have been discovered: NLRP1, NLRP2, NLRP3, AIM2 and NLRC4, of which NLRP3 has been the most studied. NLRP3 can be activated by pathogen-associated molecular patterns (PAMPs) such as bacterial liposaccharides, as well as damage-associated molecular patterns (DAMPs) including ATP [32,33]. It is believed that, in diabetes, high levels of glucose induce NLRP3 aggregation in the pancreas by activating thioredoxin-interacting protein (TXNIP) through oxidative stress [34]. Other studies have suggested that amyloid protein deposition in islet cells can also trigger IL-1β production by activating the NLRP3 inflammasome [35,36]. In retinal diseases specifically, NLRP3 activation has been reported in age-related macular degeneration (AMD) and DR [37–40]. Clinical studies have shown that patients with PDR have higher levels of all inflammasome complex components compared with controls [39]. Guo *et al*. have shown that, in ischaemia/reperfusion injuries such as retinal ischaemia, NLRP3 activation occurs and contributes significantly to the disease progression [37]. These studies show that inflammasome activation could be one of the key pathological processes in DR.

Current treatments for diabetic retinopathy and their limitations

Current treatments and preventive interventions for DR include surgical and nonsurgical therapies that target the various pathologies or biochemical pathways of the disease [41]. For over five decades, PDR and DME were primarily treated by destructive panretinal photocoagulation and vitrectomy [42]. However, these approaches can be very invasive and only target late-stage DR signs; thus, they do not affect the underlying disease aetiology. The past few decades have seen the development of less invasive drug therapies to combat the biochemical changes occurring in the disease. Table 1 summarises current treatments as well as treatments under development for DR and DME with the increasing understanding of molecular mechanisms governing the DR pathophysiology resulting in an increased discovery of new drug targets. As evident from Table 1, only four out of 36 listed drugs have been FDA-approved for DR and DME treatment to date. These include two anti-VEGF therapies (Lucentis® and Eylea®) as well as two corticosteroid-based implants (Ozurdex® and Iluvien®).

Anti-VEGF therapies

Monoclonal antibodies against VEGF revolutionized the treatment of wet AMD when they first arrived on the market over a decade ago. They are also the most commonly intravitreally administered therapy for the treatment of DR. As their name implies, these drugs target VEGF, a cytokine and growth factor implicated in the vascular breakdown that is associated with DR and other vascular diseases of the retina. By doing so, they inhibit neovascularisation of the retina, preventing tissue destruction as well as the debilitating vision loss associated with leaky, abnormal blood vessel development. For the past several years, Lucentis® (ranibizumab) and Eylea® (aflibercept) have dominated in major pharmaceutical markets, along with a third major player: Avastin® (bevacizumab), which is routinely compounded for intravitreal injection, providing a less-expensive, off-label option.

Despite the effectiveness and frequent use of anti-VEGF therapies, which have set a high bar for therapeutics entrants, a major limitation is that prolonged use can induce the onset of geographic atrophy [2,43,44] and can result in decreased choroidal thickness [44] as well as loss of retinal ganglion cells [3]. Furthermore, other studies have reported that the best corrected visual acuity (BCVA) of >50% of patients did not improve after anti-VEGF therapy with ~10% not responding to the treatment at all [45]. Poor responses to anti-VEGF therapy have been attributed to misdiagnosis of the disease, tachyphylaxis (reduced therapeutic response to a drug as a result of repeated administration) and genetic predisposition to anti-VEGF resistance. Particularly, loss of drug effectiveness after prolonged treatment has recently been recognized with ranibizumab, which appears more susceptible to tachyphylaxis [45]. Moreover, these anti-VEGF therapies are ineffective for the treatment of geographic atrophy (sometimes referred to as dry AMD). Given these limitations, alternative treatment options for DR and DME are actively being sought that will address these, and other, unmet needs.

Corticosteroids

Corticosteroids are anti-inflammatory and antiangiogenic drugs that have been reported to protect against vascular leakage by decreasing VEGF secretion and release of proinflammatory cytokines [41,46]. They are generally administered *via* intravitreal implants, such as Iluvien® (fluocinolone acetonide) and Ozurdex® (dexamethasone), that slowly release the drug over time, thus reducing the severity of steroid-associated side-effects and decreasing injection frequency. Although clinical outcomes using corticosteroid implants have been promising, their use has been limited because steroidal drugs have a tendency to increase the intraocular pressure (IOP) to levels requiring medical intervention [47]. The same study also found a correlation between prolonged corticosteroid use and the progression of cataracts. Taken together, the limitations and side effects associated with corticosteroids highlight the need for new drug targets for more-efficient treatment of DR and DME.

Role of connexin43 in diabetic retinopathy

Connexins and gap junction channels

Connexins are a multigene family of proteins with molecular weights ranging from 26 to 70 kDa. Various connexin genes have been identified in mammals and these fall into two main lineages: class I or β group (*e.g.*, connexins 26, 30, 31, 31.1 and 32); and class II or α group (*e.g.*, connexins 33, 37, 40, 43 and 46) [48]. There are 21 different connexin isotypes in the human genome with each connexin named according to its molecular weight in kDa [49]. Six connexin protein subunits oligomerise to form a hexamer known as a hemichannel or connexon (Fig. 1). A complete gap junction channel is formed by two adjacent hemichannels, one from each neighbouring cell. The docking of two hemichannels results in a pore that allows direct communication between cells. Gap junctions exist as 'plaques' that form aggregates between a few to many

TABLE 1

Currently approved and investigational treatments for DR and DME

Drug mechanism	Drug name (trade name)	Company	Listed in clinical trials for DR/DME?	FDA-approved for DR/DME?	FDA-approved for other ocular condition?
Anti-VEGF	Ranibizumab (Lucentis®)	Genentech (CA, USA)	Yes	Yes	Yes (neovascular AMD)
	Aflibercept (Eylea®)	Regeneron (NY, USA)	Yes	Yes	Yes (neovascular AMD)
	Bevacizumab (Avastin®)	Genentech (CA, USA)	Yes	No	No
	Pegaptanib (Macugen®)	Pfizer (NY, USA)	No	No	Yes (neovascular AMD)
	PAN-90806	PanOptica (NJ, USA)	Yes	No	No
TNF-α inhibitor	infliximab (Remicade®)	Janssen Biotech (PA, USA)	No	No	No
	Adalimumab (Humira®)	AbbVie (IL, USA)	No	No	No
Anti-inflammatory corticosteroids	Dexamethasone (Ozurdex®)	Allergan (NJ, USA)	Yes	Yes	Yes (uveitis)
	Fluocinolone acetonide (Iluvien®)	Alimera Sciences (GA, USA)	Yes	Yes	No
	Fluocinolone acetonide (Retisert®)	Bauch & Lomb (NY, USA)	No	No	Yes (uveitis)
Angiotensin receptor blocker	Candasartin	AstraZeneca (London, UK)	No	No	No
	Losartan	Merck (Kenilworth, UK)	No	No	No
Kallikrein inhibitor/antibody	KVD001	KalVista Pharmaceuticals (Porton Down, UK)	No	No	No
	DM199	DiaMedica (MN, USA)	No	No	No
	Ecallantide/DX-88	Dyax (MA, USA)	No	No	No
	DX-2930	Shire (Dublin, Ireland)	No	No	No
	BCX7353	BioCryst (Durham, UK)	No	No	No
	Avoralstat/BCX4161	BioCryst (Durham, UK)	No	No	No
	Icatibant (Firazyr®)	Shire (Dublin, Ireland)	No	No	No
ACE inhibitor	Enalapril	Multiple	No	No	No
	Lisinopril	Multiple	No	No	No
Tie-2 activator	AKB-9778	Akebia Therapeutics (MA, USA)	No	No	No
	AKB-9875	Akebia Therapeutics (MA, USA)	No	No	No
	AKB-9089	Akebia Therapeutics (MA, USA)	No	No	No
	HPTPβ antibody	Akebia Therapeutics (MA, USA)	No	No	No
NSAID	Ketorolac	Roche (Basel, Switzerland)	Yes	No	Yes (postoperative ophthalmic inflammation)
	Diclofenac	Multiple	Yes	No	No
	Nepafenac	Alcon (Hünenberg, Switzerland)	No	No	Yes (postoperative ophthalmic inflammation)
Antibiotic/antimicrobial	Minocycline	Multiple	No	No	No
	Squalamine	Ohr Pharmaceuticals (NY, USA)	Yes	No	No
mTOR inhibitor	Rapamycin	Pfizer (NY, USA)	No	No	No
	Everolimus (Afinitor®)	Novartis (Basel, Switzerland)	No	No	No
Posterior vitreous detachment agent	Ocriplasmin (Jetrea®)	Oxurion (Leuven, Belgium)	Yes	No	Yes (symptomatic vitreomacular adhesion)
	ALG-1001 (Luminate®)	Allegro Ophthalmics (CA, USA)	Yes	No	No
	(Vitreosolve®)	Vitreoretinal Technologies (CA, USA)	No	No	No

thousand individual gap junction channels. Connexins that form a hemichannel can be of the same (homogenous) or different (heterogeneous) isotypes. Furthermore, two hemichannels docking together to form a gap junction can be of the same (homotypic) or different (heterotypic) isotypes.

Gap junction channels are permeable to molecules up to a size of ~1 kDa or 1.5 nm in diameter, and allow rapid exchange of ions, metabolites and signalling molecules. These channels can be opened and closed by various physiological stimuli and experimental treatments. Targeted knockout models of several connexin genes have also been used to demonstrate the functional significance of these channels to normal cell physiology [50]. Studies have shown that gap junctions are important for many cell processes including electrical coupling between cells and are able to propagate coordinated responses across multiple cells. This feature is particularly important in excitable cells such as neurons where molecular and electrical cell-coupling properties are essential for function [51,52]. In the heart, connexin channels have been

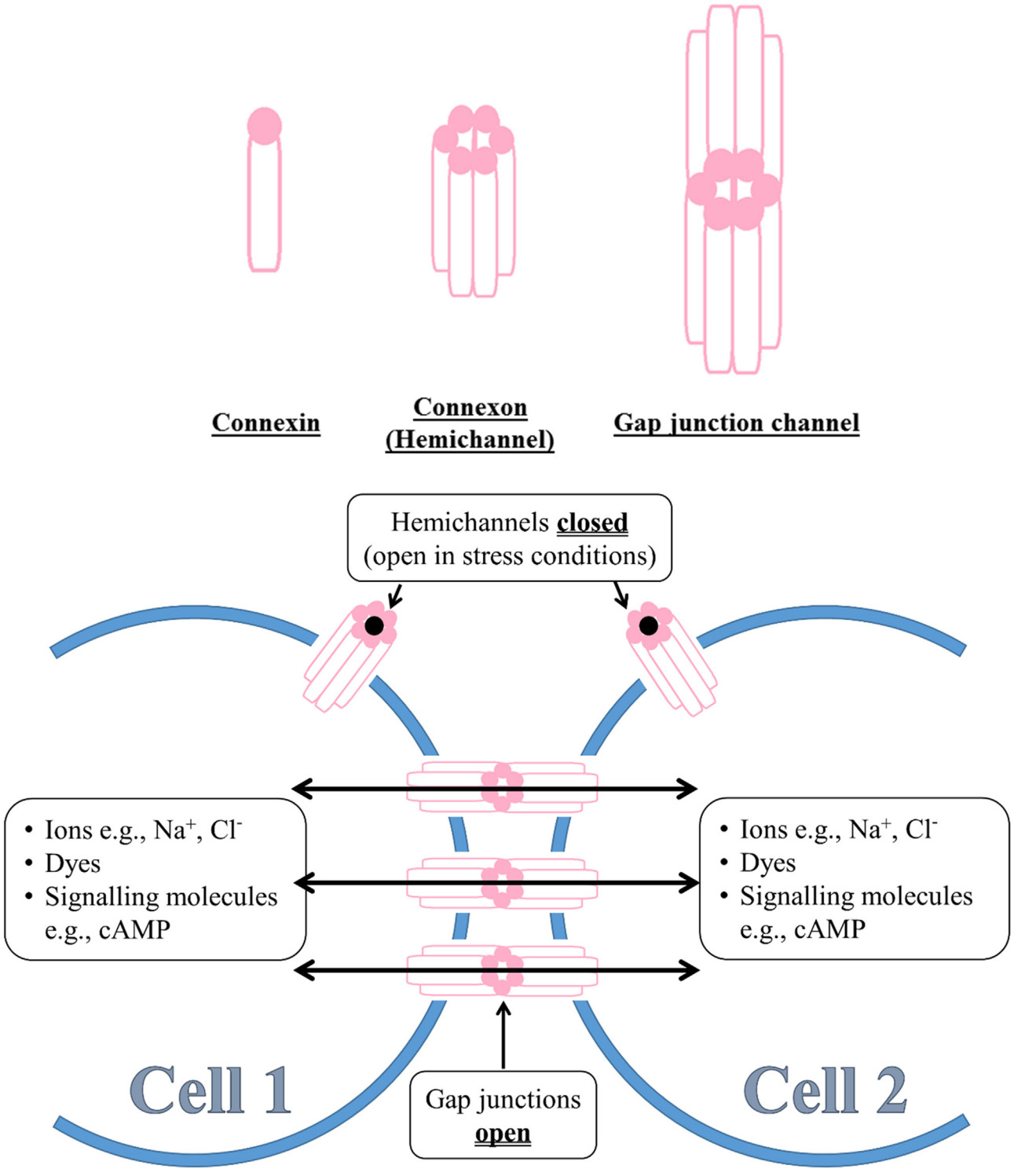

FIGURE 1

Structure of connexins, hemichannels and gap junctions. A connexin hemichannel is formed by six connexin monomers. Physiologically, hemichannels are closed but open in disease to mediate pathological events. Two connexin hemichannels from adjacent cells can dock together to form a gap junction that mediates the flow of small molecules between cells. Gap junctions are open in physiological conditions; but coupling is often, although not always, reported to be reduced following injury [62,66].

shown to coordinate embryonic development and coordinate cardiac cell depolarisation [50]. In nonexcitable cells, the biochemical and molecular coupling properties provided by gap junctions are also essential for processes such as tissue homeostasis, patterning during development and immune responses. Goliger and Paul showed that the absence of intercellular junctions can stimulate cancerous phenotypes in cells [53]. Indeed, connexin-related dysfunction is involved in many human diseases in organs such as the brain, the heart, the skin and the eye.

Of the different connexin isotypes, connexin43 is the most common in the human body. It is expressed by different cell types including astrocytes, vascular endothelial cells and cardiac cells,

and has been implicated in diseases affecting different organ systems. Particularly, connexin43 gap junction channels have been shown to play a part in inflammation, cell migration and tissue contraction [54–56]. However, although it is difficult to argue against the role of connexin43 gap junctions in injury and disease, several studies indicate that it is unopposed connexin43 hemichannels that can mediate most deleterious processes. Danesh-Meyer *et al*. explained that, with increased connexin43, there is an increase in hemichannel recruitment to the cell surface resulting in an increase in undocked hemichannels in the plasma membrane [51], and that this could be involved in paracrine and autocrine signalling between intracellular and extracellular environments [57,58]. Although these studies imply that hemichannels play a part in cell signalling and homeostasis, several studies have shown that, under normal physiological conditions, hemichannels remain closed, whereas they open during pathological situations, such as under ischaemic or hypoxic stress, to form a pathological membrane pore [59,60]. In one example, connexin43 hemichannel opening was linked to the loss of vascular endothelial cells which occurs in ischaemia [7,61]. Taken together, these studies present the possibility of studying connexin43 hemichannels as a target for the treatment of many vascular and inflammatory diseases including retinal disorders such as DR (for a recent review see [62]).

Evidence for a role of connexin43 in diabetic retinopathy

Disruption in connexin43 channel properties is thought to contribute to the pathophysiology of retinal diseases [51]. An interesting observation is that most diseases susceptible to connexin43-mediated injury, including retinal disorders, share two common pathological signs: chronic inflammation and vascular breakdown. In the retina, connexin43 is expressed by endothelial cells and astrocytes, and an increase in connexin43 expression in retinal astrocytes during cell injury has been correlated with increased propagation of calcium waves and death signals from cell to cell [51]. Calcium signalling has been shown to be involved in local blood flow regulation and induction of cytokine release, which results in BRB breakdown [63–65]. A recent study found that conditional deletion of connexin43 from astrocytes maintained a higher density of astrocytes in the hypoxic retina and promoted vascular recovery in an oxygen-induced retinopathy mouse model [66]. Another study suggested that connexin43 might mediate injury through mechanisms other than gap junction signalling. De Bock *et al*. hypothesized that the close localisation of gap junctions and tight junctions could result in a direct effect of connexin43 on cell barrier properties [67]. However, an increase in connexin43 on vascular endothelial cells has also been shown to directly affect BRB function through hemichannel-mediated rupture of endothelial cells, resulting in vascular leakage *in vivo* [51], consistent with the loss of vascular integrity typical of other inflammatory insults and diseases.

Retinal ischaemia is a key pathological feature of DR that results in loss of vascular integrity [68–70]. Wilson *et al*. showed that the resulting vascular breakdown could last for up to 2 months post injury in animal models of retinal ischaemia and reperfusion [71]. In *in vitro* endothelial cell cultures, hypoxia followed by reperfusion led to endothelial cell death which was prevented by application of connexin43 hemichannel blockers [7,61]. In *in vivo* studies where intraocular pressure was raised to induce retinal ischaemia, elevated connexin43 expression was observed in endothelial cells as early as 1 h post-injury and significant vessel die back and leak was observed starting in parallel and peaking 4 h after reperfusion [7,72]. These changes were also associated with gliosis characterised by an increase in glial fibrillary acidic protein (GFAP) expression by astrocytes. Connexin43 hemichannel upregulation therefore appears to be an early process in ischaemia that is likely to initiate inflammatory mechanisms causing vascular leakage, and persists in chronic inflammatory conditions, with hemichannel block offering benefits in the treatment of various DR signs (Table 2) [7–10,73–76].

TABLE 2

Summary of connexin43 hemichannel blockers and their effects in retinal disease models

Retinal disease	Connexin43 hemichannel blocker	Mode of delivery	Model	Therapeutic effects	Refs
Diabetic retinopathy	Peptide5	Intraperitoneal	Ischaemia/reperfusion rat model	↓Endothelial cell death ↓Inflammation ↓Vessel leak ↑Retinal ganglion cell counts	[7]
		Intravitreal	Ischaemia/reperfusion rat model	↓Inflammation ↓Vessel leak ↑Retinal ganglion cell counts	[73–75]
		Intravitreal	Diabetic retinopathy mouse model	↓Inflammation ↓Vessel leak ↓NLRP3 inflammasome activation	[9]
		–	Cell culture	↓Cytokine release ↓VEGF release ↓ATP release ↓NLRP3 inflammasome activation	[10]
Age-related macular degeneration	Peptide5	Intravitreal	Light damage rat model	↑ERG function ↓Inflammation ↑Retinal layer thickness	[8]
	Tonabersat	Intraperitoneal	Light damage rat model	↑ERG function ↓Inflammation ↑Retinal layer thickness	[76]

In contrast to the findings of increased connexin43 expression in retinal ischaemia and other chronic diseases associated with vascular breakdown and inflammation, some studies suggest that connexin43 expression decreases in diabetes. Results from *in vitro* studies where connexin43 expression was reportedly decreased as a result of hyperglycaemia were thought to correlate with a decrease in tight junction protein expression by endothelial cells, leading to increased apoptosis [77,78]. This idea is also supported by *in vivo* animal studies by this group, including, for example, work by Bobbie *et al.*, who showed that diabetes is associated with a deleterious decrease in connexin43 levels in STZ-induced diabetic mice [79]. Likewise, Tien *et al.* described a decrease in connexin43 levels in STZ-induced diabetic rats associated with increased vascular lesions in the retina [80]. In another study, Tien *et al.* evaluated the connexin43 expression pattern in human retinal donor tissues and found decreased connexin43 levels in diabetic compared with non-diabetic tissues, which was thought to lead to increased vascular lesions [81]. Although the study concluded that reduced connexin43 levels are deleterious in diabetic eyes, it is useful to consider that the diagnosis of DR in the tissues used was not documented. This distinction could be important in understanding connexin43 pathology in diabetes. Although diabetes is mainly associated with hyperglycaemia, it is now generally accepted that inflammation plays a crucial part in the development of DR. When further contemplating results from the *in vitro* and *in vivo* studies mentioned earlier, it appears that studies evaluating connexin43 pathology in the diabetic retina or retinal vascular endothelial cells exposed to high glucose have mainly utilised 'hyperglycaemia only' models that might not fully account for the role of inflammation in the disease process. This is significant because several studies using diabetic models have reported no changes in the retinal vasculature when accurate DR models should in fact present extensive changes in the retinal vasculature, similar to the human condition [82–86].

We have recently shown that clinical signs of DR are only present in diabetic mice injected intravitreally with proinflammatory cytokines whereas diabetic mice injected with saline showed no signs of vascular damage [87]. Moreover, we have compared the connexin43 expression in diabetic Akita and advanced DR Akimba mice to human donor retinas with confirmed DR diagnosis [88]. The Akimba mouse is a genetic DR model that combines the Akita mouse with a naturally occurring Ins2 gene mutation with the trVEGF029 Kimba mouse in which photoreceptors overexpress the human VEGF protein [85,86]. Rakoczy *et al.* [85] previously reported that Akimba mice had a higher prevalence of extensive retinal oedema that persisted with age compared with either parent. In our study, we found that connexin43 expression was markedly increased in human donor retinas with confirmed DR diagnosis compared with healthy controls. The pattern of connexin43 expression seen in the human tissues correlated with *in vitro* RPE cell data as well as *in vivo* Akimba results only when hyperglycaemic and inflammatory processes were

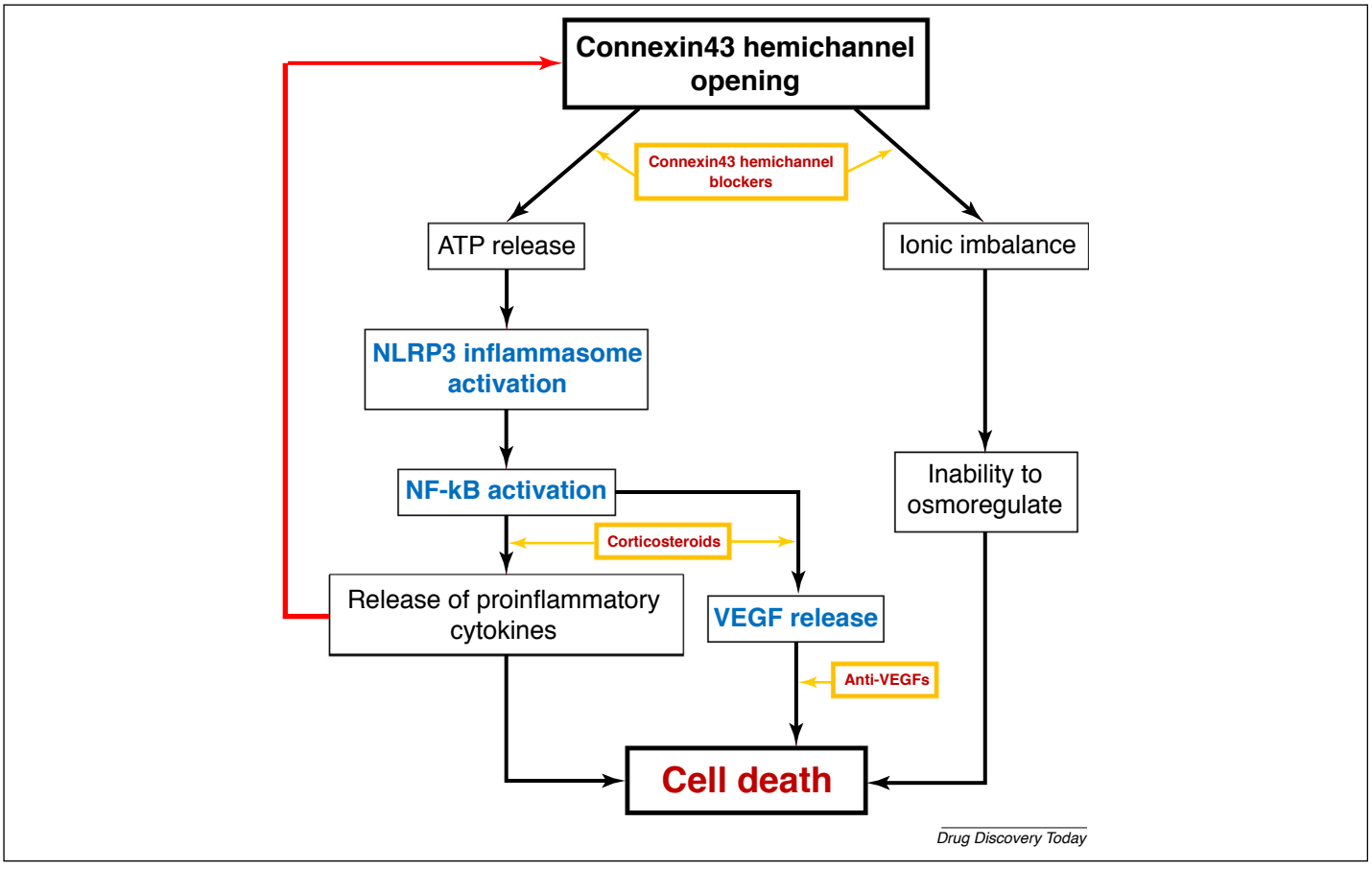

FIGURE 2

A schematic diagram showing the relationship between connexin43 hemichannel opening and the three main inflammatory pathways (blue). Sites of action for anti-VEGFs and corticosteroids are also indicated. The red arrow highlights the autocrine feedback loop between the release of proinflammatory cytokines and the opening of connexin43 hemichannels.

combined [88]. Interestingly, in diabetic mice without inflammation (Akita) there was a tendency towards decreased connexin43 expression possibly supporting other study results reporting reduced connexin43 pathology in DR using 'hyperglycaemia only' models rather than DR models with an inflammatory component. Additionally, in Akimba mice and human donor retinas with confirmed DR diagnosis, the increase in connexin43 expression correlated with leaky and damaged blood vessels, suggesting that connexin43 could play a key part in the vascular disruption seen in the disease.

These results were supported by *in vitro* cell culture studies showing that blocking of connexin43 hemichannels was effective in decreasing inflammation by interrupting an autocrine ATP feedback loop necessary for activating the NLRP3 inflammasome (Fig. 2) [10]. Furthermore, interrupting the NLRP3 inflammasome prevented VEGF release, suggesting that connexin43 hemichannel blockers can act upstream of current anti-VEGF therapies. These findings are supported by other recent *in vivo* studies where NLRP3 inflammasome proteins were found to be elevated in Akimba but not Akita and wild-type mice [40]. Activation of the nuclear factor (NF)-κβ pathway during inflammation leads to the production of pro-forms of inflammatory cytokines. To convert these cytokines from their pro-forms to the active form, pro-caspase 1 must be cleaved to active caspase 1, which occurs during NLRP3 aggregation (inflammasome complex assembly). As a result, there is a direct relationship between NF-κβ and NLRP3 pathways that is regulated by connexin43 hemichannel block. Inhibiting connexin43 hemichannels therefore has the potential to intercede early in the disease process to prevent inflammation in the first instance, and later in chronic disease to break the self-perpetuating feedback loop in the NLRP3 inflammasome pathway, a key feature of DR (Fig. 2) [10,39,40,89].

Concluding remarks

Current therapies for DR address later-stage aspects of the disease but do not target the underlying aetiology. Moreover, they have been reported to be ineffective in a large number of patients and can have significant side effects with drug resistance also reported in some patients. The increasing health and financial burden posed by diabetes and its complications means that the development of novel therapies that target upstream pathways in the DR disease process is paramount. In DR, it is believed that connexin43 hemichannel opening leads to an increase in ATP release which in turn results in activation of the inflammasome/inflammation pathways. These inflammatory pathways potentiate the DR pathology where the combination of inflammatory cytokines and high glucose levels is extremely detrimental. The evidence presented in this review discusses the therapeutic potential of connexin43 hemichannel blockers for the treatment of DR, with the prospect of breaking the inflammatory cycle that perpetuates and amplifies the disease condition. Connexin43 hemichannel blockers can restore vascular integrity (vascular normalisation) and rescue the function of retinal tissues otherwise operating within an inflammatory and ischaemic environment.

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