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Cellular Connections, Microenvironment and Brain Angiogenesis in Diabetes: Lost Communication Signals in the Post-stroke Period

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Abstract

Diabetes not only increases the risk but also worsens the motor and cognitive recovery after stroke, which is the leading cause of disability worldwide. Repair after stroke requires coordinated communication among various cell types in the central nervous system as well as circulating cells. Vascular restoration is critical for the enhancement of neurogenesis and neuroplasticity. Given that vascular disease is a major component of all complications associated with diabetes including stroke, this review will focus on cellular communications that are important for vascular restoration in the context of diabetes.

Introduction

The increasing incidence of diabetes is only the tip of the global health and social problems the disease poses. Elevated and uncontrolled blood glucose has an avalanche effect leading to debilitating complications, all which are underlined by small or large vascular disease (Association, 2014). Central nervous system (CNS) complications of diabetes including stroke and cognitive impairment are by far the least studied complications of diabetes. In addition to large vessels affected by accelerated atherosclerosis, it is increasingly recognized that small artery disease also contributes to ischemic injury of the brain (Ergul et al., 2012b). The combination of small artery disease, small infarcts and diabetes is a triple threat for the occurrence as well as the motor and cognitive recovery after stroke, which is the leading cause of disability worldwide. Therefore, better understanding of the regulation of endogenous repair mechanisms of the brain under physiological and pathophysiological conditions is fundamental to stroke research.

Vascular protection and restoration is critical for stroke recovery not only for the improvement of cerebral blood flow and blood brain barrier (BBB) integrity but also for the enhancement of neurogenesis and neuroplasticity in the angiogenic microenvironment (Ergul et al., 2012a). Our understanding of the brain repair process after stroke in diabetes is very limited. Recent studies showed that type 2 diabetes stimulates dysfunctional cerebral angiogenesis (Prakash et al., 2012; Prakash et al., 2013a). After stroke, while control animals are able to launch reparative neovascularization in both ischemic and nonischemic hemispheres, diabetic animals develop vasoregression, which is associated with poor functional recovery (Ergul et al., 2014; Prakash et al., 2013b). Impaired angiogenesis after stroke has been reported in both type 1 and type 2 diabetes (Cui et al., 2011; Ye et al., 2011). These studies showed that while vessel density is increased in the ipsilateral hemisphere 14 days after stroke, vessels fail to mature. A recent study further confirmed these results (Poittevin et al., 2014). Neovascularization, which encompasses remodeling of existing vessels, angiogenesis and barrierogenesis, is a very complex process that requires coordination of cell-to-cell interactions (Ergul et al., 2014). This cellular communication is not limited to signals among vascular cells such as endothelial cell-vascular smooth muscle cell or tip cells-stalk cells but indeed includes a network of vascular cells, surrounding resident cells of the brain including pericytes, neurons, glia, and oligodendrocytes as well as circulating blood and bone marrow cells (Figure 1). In diabetes, impaired reparative angiogenesis may be due to impaired activation of growth factors, failure in vessel maturation (lack of pericyte coverage) and reduced circulating progenitor cells (Gallagher et al., 2007). Thus, in this review, we will first define vascular repair and restoration and then systematically discuss the interactions between endothelial cells and other cell types that contribute to this process in the context of diabetes.

A. Vascular Repair and Restoration

Ischemic damage to the cerebrovasculature ignites a series of events, both local and systemic, leading to a repair process with both positive and negative consequences in the long term. Stimulation of angiogenesis and vasculogenesis can improve perfusion but chronic expression of growth factors and cytokines may lead to neointimal proliferation and atherosclerosis (Zhang and Xu, 2014). Vascular repair starts with a combination of events including arteriogenesis, angiogenesis and vasculogenesis and leads to vascular restoration with stabilized and functional vessels necessary for recovery from ischemic stroke. Immediately upon vessel occlusion, there is an increase in shear stress in the surrounding vessels, acting as a trigger for arteriogenesis. The formation of new collateral vessels from existing structures serves to maintain blood flow to the penumbral tissue and limit the damage. On a molecular level, nitric oxide (NO) has been shown to be critically important in mediating the vasodilation and formation of functional collaterals (Hoefer et al., 2006). A key feature in the persistence of the new collaterals is the recruitment of circulating monocytes/macrophages and these cells have been shown to be necessary for the maturation and growth of the collateral vessels. Endothelial and smooth muscle cell proliferation and migration follows, leading to the formation of new or expanded vessels. In the case of effective collateral formation, the larger diameter vessels relieve the shear stress and provide feedback to stop the process. With defective collateralization, as is seen in diabetes, the

stimulus for proliferation remains, making the likelihood of pathologic angiogenesis more likely (Abaci et al., 1999).

Angiogenesis genes are upregulated within minutes of the onset of cerebral ischemia, involving all cell types in the neurovascular unit (endothelial, neurons, astrocytes, etc). These cells together release growth factors including vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF), and platelet derived growth factor (PDGF) which stimulate endothelial proliferation, migration and tube formation and locally produced chemokines attract circulating and resident progenitor cells to support the process (Hermann et al., 2013). Enhanced production of proteases (MMPs) from the injured cells contributes to destroy the basement membrane and allow new vessel sprouting. Angiogenesis is followed by barrierogenesis, a process in which endothelial cells mature and establish selective permeability properties (Lee et al., 2003; Rieckmann and Engelhardt, 2003). Although most investigation of angiogenesis has been focused on the ischemic border zone after cerebral ischemia, there is emerging evidence that areas remote from the lesion also undergo angiogenic changes and this has been linked to other plasticity markers involved in recovery (Ergul et al., 2012a; Guan et al., 2011).

Vascular restoration is not only important for maintenance of perfusion of “at risk” tissue. In the aftermath of an acute ischemic stroke, neurogenesis in the subventricular zones of both hemispheres leads to the production of neuronal precursor cells (NPCs) that home to the area of injury and enhance recovery (Jin et al., 2001). Migration of NPCs occurs in close association with blood vessels, however, and it has been shown that co-transplantation of NPCs with vascular progenitor cells (VPCs) is dramatically more effective than either cell type alone in improving recovery after experimental stroke (Li et al., 2014a).

B. CNS Cell-to-Cell Interactions in Vascular Restoration

In this section, we will discuss the interaction between endothelial cells and resident cells of CNS with respect to vascular restoration starting with pericytes which are the most widely studied cells in this regard. Each subsection is organized to summarize the role(s) of a particular cell type in vascular repair/restoration, how this role is altered after stroke and finally what is known about this interaction after stroke in diabetes.

1. Pericytes—Pericytes are multifunctional cells found on pre-capillary arterioles, capillaries, and post-capillary venules (Bonkowski et al., 2011; Dalkara et al., 2011; Dore-Duffy, 2014; Hill et al., 2014; Winkler et al., 2011). They are embedded within the endothelial basement membrane (Attwell et al., 2010; Dalkara et al., 2011). While pericytes are distributed throughout the body, they are more abundant in the CNS and retina, in both of which blood barrier function is very important (Dalkara et al., 2011; Sa-Pereira et al., 2012). Their unique localization along the vasculature, while keeping in contact with astrocytes and possibly directly with neurons, strongly suggests their participation in cell-to-cell communication (Bonkowski et al., 2011; Jojart et al., 1984). Indeed, pericytes contribute to the induction and maintenance of the BBB, microvascular stability, endothelial cell survival and angiogenesis, neurovascular coupling and blood flow regulation, and clearance

of extracellular molecules by phagocytosis (Armulik et al., 2010; Bell et al., 2010; Diaz-Flores et al., 2009; Gerhardt and Betsholtz, 2003; Li et al., 2011; Ozerdem and Stallcup, 2003). Furthermore, pericytes may function as pluripotent stem cells (Bonkowski et al., 2011). Clearly, all of these actions are critical for the neurovascular repair process after ischemic brain injury and changes in the microenvironment mediated by stroke and/or diabetes can impact pericyte interactions with other cells in the CNS that ultimately affect these protective functions.

For the purpose of this review, we will focus on the dynamic role of pericytes in angiogenesis. At initiation of angiogenesis, pericytes migrate away from endothelial cells and secrete proangiogenic growth factors like VEGF-A (Bonkowski et al., 2011; Dore-Duffy and LaManna, 2007). In addition, they secrete MMPs such as MMP2 and 9 that degrade basement membrane proteins enabling the endothelial cells to sprout (Virgintino et al., 2007). Later in the process, pericytes contribute to the termination of angiogenesis and stabilization of newly formed vessels through barrierogenesis (Lee et al., 2003; Rieckmann and Engelhardt, 2003). Pericytes are recruited in response to PDGF- β secreted by endothelial cells and through the activation of PDGF-R β , they proliferate and enhance the interaction with endothelial cells (Figure 2). Seminal studies with PDGF-R β and PDGF- β knock-out mice that are basically devoid of pericytes provided key information in this field (Dalkara et al., 2011; Enge et al., 2002; Winkler et al., 2011). Together with activation of transforming growth factor- β (TGF- β) and angiopoietin-1 (Ang-1), expression of N-cadherin, integrins and gap junctions, especially connexin 43 (CX43) is stimulated. As vessel stabilization occurs, pericytes, along with astrocytes, secrete matrix proteins (Bonkowski et al., 2011; Diaz-Flores et al., 2009; Stratman et al., 2009).

The role(s) pericytes play in stroke injury and recovery is an understudied area of research. It has long been speculated that pericytes are involved in regulation of neurovascular coupling and cerebral blood flow. While it is still debatable, recent studies suggest that pericytes exhibit a prolonged contraction in response to ischemia, contributing to perfusion deficits after stroke (Yemisci et al., 2009). Hall et al also reported that ischemia mediates capillary constriction by pericytes which leads to pericyte death exacerbating the ischemic injury (Hall et al., 2014). As discussed above, pericytes may provide the initial trigger for an angiogenic response. Severity of the loss of pericytes after an ischemic event may have important implications for stroke recovery but direct evidence is lacking.

The impact of diabetes on pericyte functions in angiogenesis and microvascular integrity has been mostly studied in diabetic retinopathy as pericyte loss is a hallmark of early changes in the disease (Dalkara et al., 2011; Ejaz et al., 2008, Winkler, 2011 #21). Evidence suggests that there is a critical threshold for pericyte coverage that determines the fate of endothelial cells (Ejaz et al., 2008; Winkler et al., 2011). When pericyte number is decreased, there is a proliferative response leading to pathological angiogenesis. When pericyte loss exceeds 50%, endothelial cells start undergoing apoptosis and vasoregression. A recent study suggested that a potential link between diabetes and pericyte deficiency may be due to phosphorylation of PDGF-R β under hyperglycemic conditions which renders the receptor less responsive to PDGF- β stimulation (Gerald et al., 2009). It has also been suggested that angiopoietin-2 (Ang-2) may contribute to pericyte loss in diabetes. Ang-2 is increased

in retinal endothelial cells as well as in the vitreous of patients with diabetic retinopathy (Park et al., 2014). A recent study showed that Ang-2 induces pericyte apoptosis in the retina by upregulation of $\alpha 3\beta 1$ integrin signaling. Studies also suggest that CX-43 expression, another important protein for endothelial-pericyte communication, is decreased in diabetes (Li et al., 2003).

The effect(s) of diabetes on CNS pericytes are not fully understood. We recently showed that diabetes promotes pathological angiogenesis in the brain and this is associated with decreased pericyte coverage (Figure 3) (Prakash et al., 2012). Our studies indicate that Ang-2 expression is increased in brain microvascular endothelial cells isolated from these animals which may contribute to decreased pericytes in this model (unpublished data). It is highly possible that diabetes can mediate PDGF-R β phosphorylation or decreased CX-43 expression in CNS pericytes as reported in the retina (Figure 2).

As discussed above, vascular repair is impaired and there is substantial vasoregression after stroke in diabetes. Our results suggest that this vascular loss occurs in the presence of VEGF and it involves peroxynitrite-mediated apoptosis of endothelial cells (Abdelsaid et al., 2014). The role of pericytes in this response is practically unknown. In light of the reports that pericyte density is an important determinant of the vascular reparative response, one can speculate that extensive loss of pericytes contributes to post-stroke cerebral vasoregression in diabetes.

2. Glial Cells—The most prominent cell found in the CNS is the astrocyte. A strong body of literature indicates that astrocytes not only provide structural support for neurons and endothelial cells but also are involved in the regulation of neuronal metabolism and cerebral blood flow (CBF), and enable synapse formation and BBB integrity via direct contact with endothelial cells with their end-feet (Barres, 2008; Liebner et al., 2011; Ransom and Ransom, 2012; Ullian et al., 2004). One reason they may be able to accomplish these tasks is they coordinate neurovascular communication with their numerous processes. We will briefly discuss the role(s) of astrocytes in angiogenesis and barrierogenesis. Many angiogenic growth factors such as VEGF-A, BDNF and insulin like growth factor-1 (IGF-1) are secreted by astrocytes (Fulmer et al., 2014; Lee et al., 2009; Li et al., 2014d; Lopez-Lopez et al., 2004). An earlier study reported that meteorin, a protein highly abundant in astrocytes, stimulates expression and secretion of antiangiogenic proteins thrombospondin-1 (TSP-1) and TSP-2 in an autocrine manner which then inhibit angiogenesis by the surrounding endothelial cells (Park et al., 2008). It appears that astrocytes and pericytes are both involved in the formation and maintenance of capillary like structures by endothelial cells (Itoh et al., 2011). Studies have also shown that astrocytes are necessary for barrierogenesis and directly regulate factors modulating BBB formation, such as tight junction proteins, SSeCKS, Ang-1 and TGF β (Bonkowski et al., 2011; Dore-Duffy and LaManna, 2007; Dore-Duffy, 2014; Garcia et al., 2004; Lee et al., 2003; Rieckmann and Engelhardt, 2003; Rubin et al., 1991).

Astrocytes exhibit a multimodal neurovascular response when confronted with insults such as diabetes and stroke. The role of astrocytes in the neurovascular repair process after stroke is complex and controversial. While reactive astrocytes can contribute to glial scar formation and secrete factors that inhibit axonal regeneration, it is also possible that reactive astrocytes

protect neurons by taking up excess glutamate, secreting neurotrophic factors and providing a barrier that limits expansion of damage (Faulkner et al., 2004; Hansson and Ronnback, 2003; Li et al., 2014c; Liu et al., 2014; Mazzanti et al., 2001). A recent study provided evidence that attenuated astrocytic reactivity can impair axonal remodeling and functional recovery (Liu et al., 2014). A central role for reactive astrocytes in mediating CNS plasticity and recovery after stroke was recently reviewed (Li et al., 2014c) but how astrocytes contribute to vascular plasticity is not fully understood. Interestingly, angiostatic proteins TSP-1 and TSP-2, which also have synaptogenic properties, are increased mainly in astrocytes after stroke (Liau et al., 2008; Lin et al., 2003). It has been postulated that upregulation of these proteins are responsible for the resolution of postischemic angiogenesis (Liau et al., 2008; Lin et al., 2003) but a clear role in vascular repair is not known.

Supporting the concept that reactive astrocytes contribute to the recovery process, Jing et al. reported that while ischemia caused a proliferation of astrocytes in the penumbra region in control animals, there was a significantly lower amount of reactive astrocytes in diabetic animals which display worse outcome (Jing et al., 2013). This decrease in astrocytes was associated with greater BBB damage and the brain repair process was stunted. They also identified regions where astrocyte infiltration was greater, and there was a significant improvement in vascular and neural reconstruction. Similarly, Kumari and colleagues reported that robust increases in astrocytic and microglial activation observed in normal mice are blunted in diabetic mice after ischemic injury and this is accompanied by decreased and delayed expression of IGF-1 by these cells (Kumari et al., 2007). As mentioned above, in addition to its neuroprotective properties, IGF-1 also stimulates angiogenesis. It is known that post stroke angiogenesis is impaired in aged animals and is associated with concurrent decreases in IGF-1 (Ingraham et al., 2008; Sonntag et al., 1999). Modulation of IGF-1 may be another mechanism by which glial cells contribute to repair after stroke, especially in the diabetic setting. In contrast to these studies, we found amplified astrocyte reactivity in their type 2 diabetic model with an abundance of thin processes and a reduced soma size after stroke (Prakash et al., 2013b). These changes in astrocyte morphology were associated with vasoregression and poorer outcomes in diabetes. These differences in the activity levels of astrocytes may stem from the use of different models of diabetes with varying disease severity as well as the severity of the ischemic injury. Nevertheless, these studies suggest that modulation of astrocytic reactivity may contribute to the impairment of vascular repair in diabetes. Interestingly, as pointed out by Liu and colleagues, there are different forms of astrocytes in the CNS (Liu et al., 2014). Differential role(s) of these astrocytes in vascular repair deserves further investigation.

Microglia are bone marrow-derived macrophages that reside in the CNS. In addition to their immune functions, they closely interact with the vasculature. Indeed, studies with different *in vivo* models in which microglia were lacking demonstrated a critical role for microglia in angiogenesis in the brain (Arnold and Betsholtz, 2013; Fantin et al., 2010). A recent report provided evidence that conditioned medium from BV2 microglial cells promotes angiogenesis via upregulation of angiogenic factors ephrin-A3 and ephrin-A4 in endothelial cells. Moreover, they showed that tumor necrosis factor (TNF)- α secreted from microglia

mediates this effect (Li et al., 2014b). While studying the effect of epidermal growth factor treatment of neurogenesis in the supraventricular zone (SVZ), Lindberg et al showed critical role of microglia in the angiogenic response in this neurogenic niche (Lindberg et al., 2014).

Microglia are activated after stroke. The pattern of activation depends on the localization with respect to infarct and involves polarization of microglia either into a protective M2 alternatively activated phenotype or proinflammatory M1 classically activated phenotype (Lee et al., 2014). Collectively, the balance can determine the impact of microglia on neurovascular repair. In this regard, information on the role of microglia in angiogenic response after stroke is limited. It has been reported galectin-3 (Gal-3), an angiogenic protein that is primarily localized to microglia, is increased after ischemic injury and augments endothelial proliferation and microvessel density (Lalancette-Hebert et al., 2012; Yan et al., 2009). Yan and colleagues recently reported that Gal-3 secreted by BV2 microglial cells enhances angiogenic potential of neighboring endothelial cells via activation of integrin-linked kinase signaling (Wesley et al., 2013). Microglia and endothelial cell interactions after stroke in diabetic setting are even less understood. As discussed above, Kumari and colleagues reported blunted astrocyte/microglial activation after ischemic injury in diabetes and this was associated with decreased and delayed secretion of TNF- α and IGF-1 (Kumari et al., 2007). Since microglial TNF- α has been shown to enhance angiogenesis (Li et al., 2014b), it is possible that this may be another mechanism contributing to impaired repair and vasoregression after stroke in diabetes.

3. Neurons—Nerves and blood vessels are arguably the backbone of communication throughout living systems. As such, it is not surprising that neurons and endothelial cells are in a bidirectional communication to orchestrate the repair process after ischemic injury. It is increasingly recognized that neurogenesis and synaptogenesis occur in concert with angiogenesis in experimental models of recovery after ischemic brain injury (Ergul et al., 2012a; Navaratna et al., 2009; Xiong et al., 2010). A recent study demonstrated that endothelial cells transplanted into the brain promote vasculogenesis and enhance neurogenesis further providing support for this concept (Ishikawa et al., 2013).

One of the most studied growth factor involved with both neuronal and endothelial cell survival is VEGF-A. VEGF-A and its receptor VEGFR2 has been proven to aid in the migration and differentiation of endothelial cells as well as in vasculogenesis, and angiogenesis (Dumont et al., 1995; Gerhardt et al., 2003; Kearney et al., 2004; Ruhrberg, 2003; Shalaby et al., 1995). Originally isolated from the vasculature, it is also known that neurons express VEGF and its receptors. There are many *in vitro* and *in vivo* studies indicating how VEGF-A regulates neuronal proliferation, size, survival and guidance (Bocker-Meffert et al., 2002; Erskine et al., 2011; Jin et al., 2002; Khaibullina et al., 2004; Rosenstein et al., 2003; Ruiz de Almodovar et al., 2010; Ruiz de Almodovar et al., 2011; Silverman et al., 1999). A classic and powerful example of the interaction between angiogenic and neurotrophic effects of growth factors is the song bird studies in which increased VEGF-induced angiogenesis has been shown to promote proper development of young neurons in the higher vocal center via the release of BDNF (Hartog et al., 2009; Louissaint et al., 2002; Rasika et al., 1994).

Several other growth factors have been implemented in this bidirectional communication. BDNF is synthesized by both neurons and endothelial cells and has neurotrophic and angiogenic effects (Alhusban et al., 2013; Kermani et al., 2005). Endothelial cell conditioned media can significantly increase the viability of neurons after various insults including hypoxia, amyloid- β challenge, oxidative stress, or endoplasmic reticulum stress and this is mediated by BDNF (Guo et al., 2008). An interesting recent study provided evidence that neuronal TGF β is also involved in brain angiogenesis. Neuronal knock-out of TGF β receptor 2 altered the profile of proangiogenic factors secreted by neurons such as VEGF, IGF-1 and FGF. These changes were associated with atypical blood vessel formation (Hellbach et al., 2014). Nerve growth factor (NGF) is another neurotrophin with opposing endothelial actions. While tropomyosin-related kinase A (TrkA) receptor activation by NGF promotes endothelial integrity and averts pericyte damage, NGF precursor protein, proNGF, mediates endothelial cell death by p75^{NTR} (Hammes et al., 1995; Kim et al., 2004).

After stroke, numerous studies has shown that VEGF and its receptors are upregulated not only in endothelial cells but in neurons and astrocytes as well (Beck et al., 2002; Brockington et al., 2006; Forsythe et al., 1996; Hai et al., 2003; Jin et al., 2000; Lennmyr et al., 1998; Lennmyr et al., 2005; Shin et al., 2008; Tolosa et al., 2008; Wang et al., 2004) and associated with improved neurogenesis, angiogenesis and functional outcome (Jin et al., 2002; Li et al., 2009; Wang et al., 2007).

Axonal guidance proteins have been shown to contribute to both endothelial and neuronal repair. One such protein is netrin-1 (Lu et al., 2011; Lu et al., 2012; Sun et al., 2011; Wu et al., 2008). Studies using netrin-1 deficient mice showed vast blood vessel impairment (Park et al., 2004). Following stroke, netrin-1 reduces infarct size, attenuates neuronal death, strengthens functional outcome, prompts neuronal migration, and fosters angiogenesis. Another neuronal guidance molecule that affects angiogenesis is semaphorin 3A (Sema3A). In ischemic retinopathies, Sema3A binds to neuropilin-1 (Nrp-1) and inhibits endothelial cell motility and angiogenesis (Joyal et al., 2011; Klagsbrun and Eichmann, 2005; Mamluk et al., 2002; Miao et al., 1999). However, the roles of these axonal guidance proteins in diabetes and especially in recovery after stroke are not known.

Our understanding of neuron-endothelial cell-astrocyte interactions that can affect neuronal and vascular repair after stroke in diabetes is very limited. While we know that diabetes worsens functional recovery and this is associated with cerebral vasoregression and infarct expansion (Ergul et al., 2014; Li et al., 2013; Prakash et al., 2013b), underlying mechanisms remain unknown. We have evidence that this vasoregression occurs in the presence of VEGF (Abdelsaid et al., 2014) suggesting that microenvironment is critical for protective and reparative functions of growth factors involved in recovery. Evidence suggests that oxidative and nitrative stress diverts survival effects of VEGF to a proapoptotic pathway in endothelial cells in diabetes. Whether VEGF signaling is affected in a similar way in neurons remains to be determined. Microenvironment can also affect the generation of restorative growth factors. For example in the eye, oxidative stress inactivates MMP-7, which cleaves the precursor protein, proNGF, to its mature form, NGF, resulting in excess proNGF which causes neuronal and vascular injury in the retina via p75^{NTR} signaling (Al-Gayyar et al., 2011; Bruno and Cuello, 2006). A similar paradigm may exist for BDNF in

diabetes. Using an ELISA which detects both pro and mature forms, we reported greater BDNF levels in the brains of diabetic GK rats. Yet, the outcome of stroke was poor in this model (Li et al., 2010; Li et al., 2013). We now have data that proBDNF and p75^{NTR} levels are greater at baseline and increased even further after stroke in diabetic animals. A recent study reported that proBDNF negatively regulates neuronal remodeling (Yang et al., 2014). Another change with regard to BDNF effects may involve TrkB receptors, which are activated by BDNF and neuroprotective. A recent study showed that advanced glycation end-products degrade neuronal TrkB receptor via upregulation of MMP-9 (Navaratna et al., 2013). It is of great interest to determine the localization of these signals and whether these mechanisms contribute to vasoregression and/or impaired neuronal repair after stroke in diabetes.

4. Oligodendrocytes—Oligodendrocytes are known for myelinating axons and therefore play an important purpose in signal transmission among the neurovascular network. However, their contribution to neurovascular repair and interaction with other cells in this process is far less understood. Once stroke occurs, there is a rapid loss of oligodendrocytes due to ischemia-related oxidative stress, glutamate excitotoxicity and depolarization (Arai and Lo, 2009; Dewar et al., 2003; Micu et al., 2006). Oligodendrocyte progenitor cells work rapidly to restore demyelinated axons (Gensert and Goldman, 1997). Endothelial cells have even been shown *in vitro* to promote the production and sustenance of oligodendrocyte progenitor cells by Akt and Src signaling pathways by releasing the trophic factors such as bFGF and BDNF (Arai and Lo, 2009). Recent studies by Jiang et al. also show that oligogenic activity and oligodendrocyte progenitor maturation typically occur in the peri-infarct basal ganglia moderately concurrent with increased vessel density. Another interesting finding by Arai K et al. is that oligodendrocyte progenitor cell may not always play the role of the protagonist during injury recovery since they release MMP-9 (Arai and Lo, 2009). MMP9 is linked to BBB breakdown during white matter injury thus potentially aggravates disease states, once again proving that glial activation can be either advantageous, by protecting healthy cells and aiding in the restoration of injured ones, or deleterious, by exacerbating the disease state and impeding regenerative growth (Giaume et al., 2007; Heneka et al., 2010).

Astrocytes are also involved in the myelination process as increased astrocyte infiltration directly correlated with increased remyelination (Jing et al., 2013). This is not surprising considering oligodendrocyte myelination is promoted by astrocyte-secreted cytokine leukemia inhibitory factor (Ishibashi et al., 2006). The interesting discovery from Li PA's group is that, while stroke conditions induce demyelination and inhibit astrocyte proliferation and mobility, diabetes worsens this demyelination and prolongs recovery (Jing et al., 2013).

C. Vascular Restoration and Interactions with Circulating Cells

In this section, we will discuss the role of circulating cells in pathogenesis and recovery after diabetic stroke. We will also highlight their possible role in neovascularization and how it could be altered in the diabetic milieu. Circulating cells comprise all cell types in the systemic circulation that can infiltrate the brain after ischemic stroke and/or modify stroke

outcome. This includes immune cells and stem/progenitor cells. In addition, we will also discuss microparticles that are secreted by circulating cells and have been shown to impact stroke outcome.

1. Immune cells—Several studies have shown that diabetes affects the immune response. In one of the earlier studies, Panes *et al.* investigated the systemic immune cell recruitment after peripheral ischemia reperfusion injury in diabetes (Panes et al., 1996). They compared leukocyte endothelial cell adhesive interaction in the mesenteric venules of control, hyperglycemic and streptozotocin (STZ) induced type 1 diabetic rats. Diabetic rats showed increased number of rolling but not adherent or emigrating leukocytes at baseline. Ischemia-reperfusion elicited larger increases in leukocyte adhesion and emigration in diabetic but not acutely hyperglycemic rats. These effects were mediated through CD11/CD18–ICAM-1 interactions (adhesion) and P-selectin (rolling). Other studies showed decreased membrane fluidity (Masuda et al., 1990), and enhanced superoxide production (Freedman and Hatchell, 1992) by granulocytes isolated from diabetic animals. Clinically, neutrophils isolated from diabetic patients produced significantly greater amounts of superoxide anions, which correlated negatively with patients' glycemic control (Karima et al., 2005; Wierusz-Wysocka et al., 1987). Moreover, neutrophils isolated from healthy adults are activated under elevated glucose levels (Kummer et al., 2007). Similarly, monocytes isolated from type 2 diabetic patients showed a pro-inflammatory profile while monocytes from healthy volunteers showed increased secretion of the inflammatory cytokine, TNF- α when cultured under high glucose conditions (Gonzalez et al., 2012). These findings demonstrated that immune cells are activated in the diabetic milieu and with high glucose levels. Thus, diabetic subjects might be more prone to immune mediated tissue injury after stroke.

Based on the previously mentioned studies, it is conceivable that diabetes affect the immune response to stroke as well. While the effect of stroke on immune cells has been examined in different studies, reports studying the immune cell function in diabetic stroke are only beginning to emerge. These studies are listed in Table 1. As obvious from the table, the studies are few and mostly recent (only came out in the last 4 years). Interventional studies using circulating cells in diabetic stroke are listed in Table 2. The effect of diabetes on systemic cells' functions after stroke is highlighted in Figure 4.

Neutrophils, the most abundant type of granulocyte, are among the first cells to be recruited to the brain after stroke (Jin et al., 2010) and have been associated with worsened outcome clinically (Kim et al., 2012; Price et al., 2004) and experimentally (Matsuo et al., 1994; Shiga et al., 1991). Ritter et al. examined neutrophil adhesion in type 2 diabetes and stroke using Zucker Diabetic Fatty (ZDF) rats subjected to 2 h middle cerebral artery occlusion (MCAO) (Ritter et al., 2011). Diabetic rats showed increased neutrophil adhesion and decreased shear rate. This was associated with increased expression of CD11b (a measure of neutrophil activation) and soluble intercellular adhesion molecule (sICAM – a ligand for neutrophil adhesion) and correlated with increased inflammatory genes expression, thus leading to poor stroke outcome.

Among the chemotactic factors involved in attracting immune cells to the site of injury after stroke is monocyte chemotactic protein-1 (MCP-1). MCP-1 gene polymorphism was

strongly associated with susceptibility to ischemic stroke. This polymorphism tended to be higher in diabetic patients (Buraczynska et al., 2010). Kim et al. have recently examined how diabetes alters the inflammatory response and stroke outcome (Kim et al., 2014). Using diet induced type 2 diabetes in mice, the authors showed an increase in MCP-1 in peritoneal immune cells collected from diabetic mice before and after stroke. Interestingly, they found a blunted inflammatory response in the diabetic brain before stroke and early at 6 h post-stroke manifested by decreased MCP-1 RNA compared to controls. This correlated with attenuated lipopolysaccharide (LPS)-stimulated inflammatory response in diabetic macrophages suggesting a deregulation of the inflammatory cascade in diabetic animals. Despite this, diabetic animals showed exacerbated ischemic brain injury.

While immune cell recruitment has been shown to worsen stroke outcome, it can also play a beneficial role through promoting neovascularization. As mentioned earlier, monocyte/macrophage recruitment is necessary for arteriogenesis and collateral vessel maturation. MCP-1 and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) have been shown to increase arteriogenesis and collateralization after femoral artery occlusion through increased monocyte/macrophage survival (Buschmann et al., 2001; Ito et al., 1997). Similarly, GM-CSF enhances arteriogenesis and leptomeningeal collateral growth in the hypo-perfused rodent brain through monocyte/macrophage recruitment (Buschmann et al., 2003; Todo et al., 2008). Clinically, different monocyte subtypes were associated with either tissue injury or repair and angiogenesis after stroke (Urta et al., 2009). In diabetes, peripheral neovascularization and collateral vessel development is impaired secondary to reduced monocyte recruitment. This is believed to be due to defective VEGF receptor signaling in these cells (Carr et al., 2011; Tchaikovski et al., 2009; Waltenberger, 2001). However, how diabetes affects the immune cell mediated neovascularization in the brain is yet to be discovered.

2. Stem/progenitor cells—Different stem/progenitor cells have been shown to play a beneficial role in ischemic stroke recovery. These cells are not terminally differentiated and include endothelial progenitor cells, and bone marrow stromal cells.

a. Endothelial progenitor cells: Circulating endothelial progenitor cells (EPCs) represent a specific type of bone marrow derived immature cells that have the ability to differentiate to endothelial cells (Yoder, 2012). EPCs can be isolated from bone marrow, cord or peripheral blood. EPCs express surface markers similar to hematopoietic and endothelial cells and are recruited to the endothelium following hypoxia or ischemia. EPCs characterization is difficult and they are generally identified by staining for CD34, CD133, or VEGFR2 using flow cytometry. These cells play an important role in angiogenesis and vascular repair (Zhao et al., 2013). These functions can be impaired under the oxidative environment of diabetes.

The clinical importance of endothelial progenitor cells in endogenous vascular repair has been highlighted early on in coronary artery disease (Schmidt-Lucke et al., 2005; Werner et al., 2005). Similarly, ischemic stroke patients with increased circulating EPCs levels show better functional outcome at 3 months (Marti-Fabregas et al., 2013; Sobrino et al., 2007), while fewer number of EPCs on admission is an independent risk factor for poor outcome at 6-month (Tsai et al., 2014). Acute statin therapy or pretreatment, which are known to

improve stroke outcome, have been shown to increase circulating EPCs levels in the first week after stroke (Marti-Fabregas et al., 2013; Sobrino et al., 2012).

Experimentally, EPCs have been shown to participate in neovascularization, endothelial repair and vascular restoration after ischemic stroke. A study by Zhang et al. investigated the contribution of EPCs to neovascularization after stroke (Zhang et al., 2002). Using embolic stroke mouse model and bone marrow transplantation, they could show the incorporation of EPCs in the newly formed vessels in the ischemic border zone. This highlights the contribution of endogenous EPCs to ischemic recovery through vasculogenesis. Interestingly, administration of EPC conditioned media achieved similar proangiogenic response and functional recovery suggesting that EPCs mediate some of their actions via growth factors secretion (Rosell et al., 2013).

EPCs are reduced and dysfunctional under diabetic milieu. Diabetic patients with stroke and atherosclerotic intracranial artery stenosis have reduced circulating EPCs compared to respective non-diabetics (Liu et al., 2013). Experimentally, type 2 diabetic db/db mice show reduced angiogenic response to bone marrow-derived EPC infusion after stroke (Bai et al., 2014). Taken together, EPC-mediated angiogenic and vascular restorative actions seem to be impaired in diabetic stroke.

b. Bone marrow stromal cells: Exogenously administered bone marrow stromal cells (BMSCs) have been shown to enhance regeneration and functional recovery after stroke through promoting angiogenesis and vascular stabilization (Chen et al., 2001; Zacharek et al., 2007). When administered to type 1 diabetic rats after stroke and followed up for 14 days, BMSCs therapy could not elicit functional recovery and was associated with higher mortality, BBB leakage, and brain hemorrhage. BMSCs treatment also increased cerebral arteriosclerosis after stroke in these rats as manifested by internal carotid artery neointimal formation and cerebral arteriole narrowing/occlusion (Chen et al., 2011b). In a subsequent study, the authors adopted a combination treatment of BMSC with 40 mg/kg of Niaspan administered daily for 14 days after stroke (Yan et al., 2013). Niaspan is a prolonged release formulation of niacin (vitamin B3), which has anti-atherosclerotic properties by increasing high-density lipoprotein (HDL) levels and decreasing low-density lipoprotein (LDL) levels. The combination treatment reduced the atherosclerotic effects of BMSCs in diabetic rats and enhanced white matter remodeling and synaptic protein expression (Ye et al., 2013). However, it did not improve functional outcome. The same group later tested human umbilical cord blood cells (HUCBCs) under the same experimental conditions (Yan et al., 2014). They found that HUCBCs could improve functional outcome and enhanced white matter and vascular remodeling.

3. Microparticles—Microparticles (MPs) are membrane bound vesicles (100 to 1000 nm in diameter) that are released by budding from different cell types (Mause and Weber, 2010). They are present physiologically, with 70 to 90 % of circulating MPs being derived from platelets. MPs are considered a form of communication between cells since they enclose different signaling molecules, and thus, can influence the function and phenotype of recipient cells. Moreover, they can be tracked to their parent cells through analysis of

surface antigens. MPs can be increased under different pathological conditions such as autoimmune disorders, atherosclerosis, cancers, and infection (Mause and Weber, 2010).

Platelet derived MPs (PMPs) are elevated acutely after cerebral infarction and correlate with the presence of intracranial atherothrombotic lesions (Kuriyama et al., 2010). Apart from the procoagulative properties of PMPs, these particles are thought to contain growth factors that may promote brain cells survival and neurovascular restoration. Experimentally, PMPs were shown to be involved in the neuroprotection conferred by remote ischemic conditioning after stroke (Shan et al., 2013). Similarly, exogenous administration of platelet derived MPs was shown to induce angiogenesis and neurogenesis after permanent MCAO in rats and was associated with behavioral recovery at 90 days in rats (Hayon et al., 2012a). *In vitro*, treatment of neural stem cells with platelet derived MPs increased their survival and led to larger neurospheres. Moreover, the treatment also increased the differentiation potential of neural stem cells (NSC) to glia and neurons. These effects were attributed to various growth factors contained within the PMP (Hayon et al., 2012b). Interestingly, in these studies, ischemia was induced using suture occlusion and it would be very interesting to examine the role of microparticles in the more clinically relevant embolic model to assess their procoagulative potential.

Type 2 diabetic patients have elevated levels of MP which correlate with diabetic complications (Tsimerman et al., 2011) such as vascular dysfunction (Feng et al., 2010), diabetic nephropathy (Omoto et al., 1999), diabetic retinopathy (Ogata et al., 2005; Ogata et al., 2006), myocardial infarction (Morel et al., 2004), atherosclerosis (Nomura et al., 1995), coronary artery disease (Nomura, 2009), and possibly stroke. There is one preclinical study in which Chen *et al.* (Chen et al., 2011a) investigated the function of circulating endothelial progenitor cells (cEPCs) and cellular membrane microparticles (MPs) in diabetic stroke using db/db mice, a genetic model of type 2 diabetes with a point mutation in the leptin receptor. Bone marrow-derived EPCs were reduced in db/db mice and dysfunctional as measured by reduced tube formation ability *in vitro*. In contrast, the levels of EPC-derived MPs were increased in these mice after stroke and correlated with fasting blood glucose and cerebral ischemic damage. The same group investigated the effect of EPCs priming with chemokine receptor type 4 (CXCR4) using adenovirus. The CXCR4-primed EPCs therapy was able to reduce the ischemic brain damage and promote repair in db/db mice (Chen et al., 2012).

Conclusions

The reasons why years of stroke research has failed to identify new therapeutic approaches are multifactorial. However, there are 2 major points that all researchers agree upon. First, stroke research should not only focus on neurons and look at repair and recovery mechanisms from a broader perspective. With the development of neurovascular unit concept, there is more intense investigation of cell-to-cell communications within the ischemic and peri-infarct area to meet this need. Recent studies suggest that nonischemic hemisphere also contributes to the repair and recovery following stroke (Ergul et al., 2012a; Guan et al., 2011; Prakash et al., 2013b). As summarized above, circulating cells are also involved in this response. Collectively, we propose that cellular communications leading to

recovery are very complex and include whole body responses. Second, most therapeutic targets have been identified in otherwise healthy and young animals without comorbidities that are common to stroke patients. Most if not all studies discussed in this review used only male animals. Diabetes is the most rapidly increasing comorbidity in acute ischemic stroke. As we move forward to better understand the role(s) of cell-to-cell communications in neuronal repair and vascular restoration, diabetes and sex should be included in experimental models.

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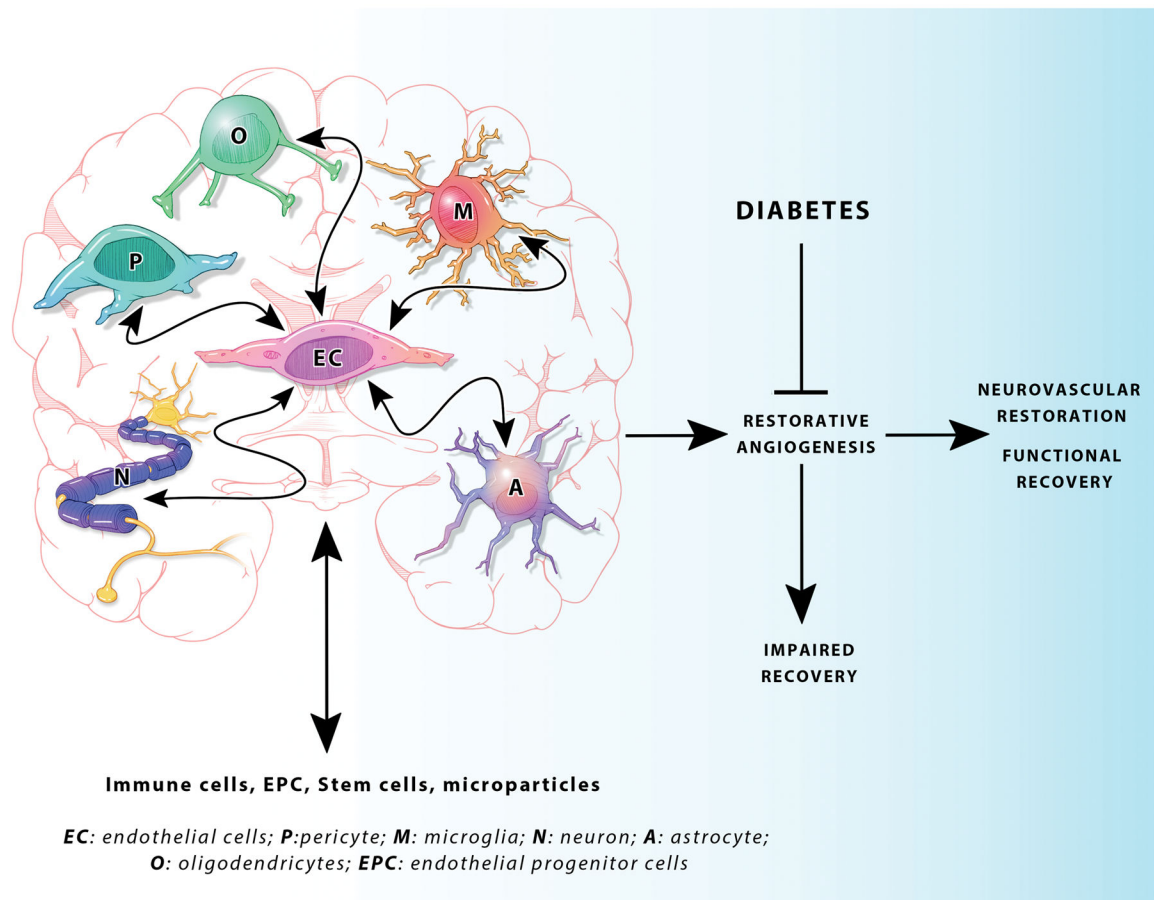


Figure 1. A schematic representation of the bidirectional interactions between various CNS and peripheral cell types that contribute to neurovascular repair after stroke
 Diabetes impedes vascular restoration and functional recovery.

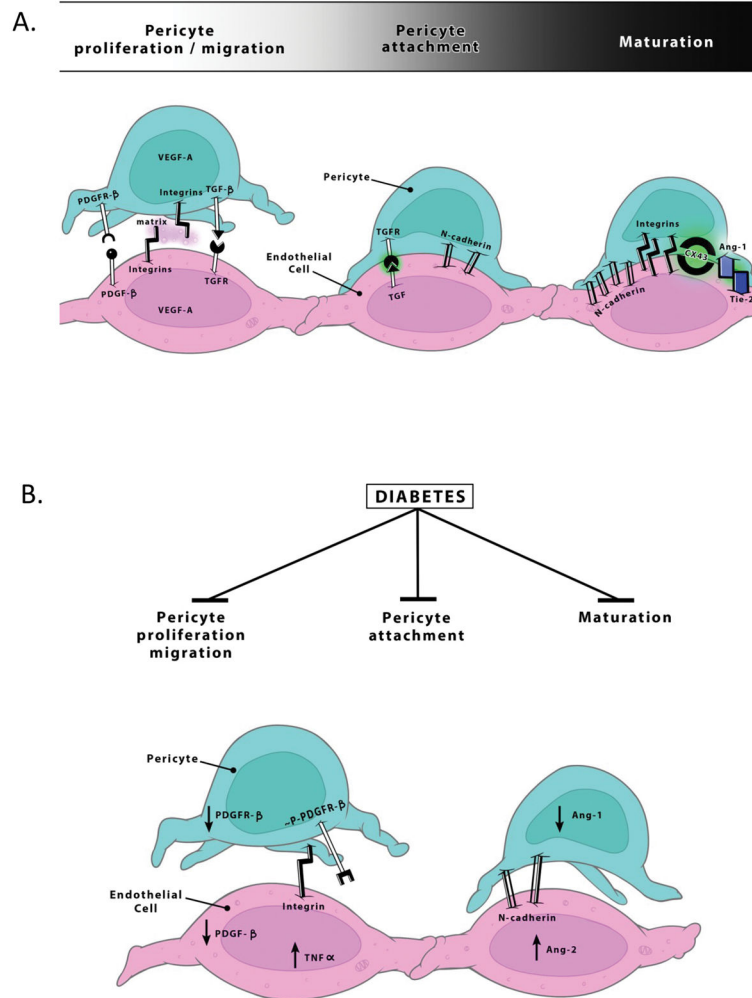


Figure 2. Possible mechanisms contributing to weakened pericyte-endothelial cell interactions in diabetes

A. At the initiation of angiogenesis, pericytes migrate away from endothelial cells and secrete proangiogenic growth factors like VEGF-A. Pericytes are later recruited in response to PDGF- β secreted by endothelial cells and through the activation of PDGFR- β , they proliferate and enhance the interaction with endothelial cells. Pericytes contribute to the termination of angiogenesis and stabilization of newly formed vessels through barrierogenesis. Activation of TGF- β and Ang-1, stimulates expression of N-cadherin, integrins and gap junctions, especially connexin 43 (CX43). As vessel stabilization occurs, pericytes, along with astrocytes, secrete matrix proteins. B. In diabetes, pericyte recruitment, attachment and maturation are impaired. Increased phosphorylated pPDGFR- β , decreased PDGFR- β expression and or alterations in Ang-1 and Ang-2 signaling may contribute to loss of communication between these two cell types.

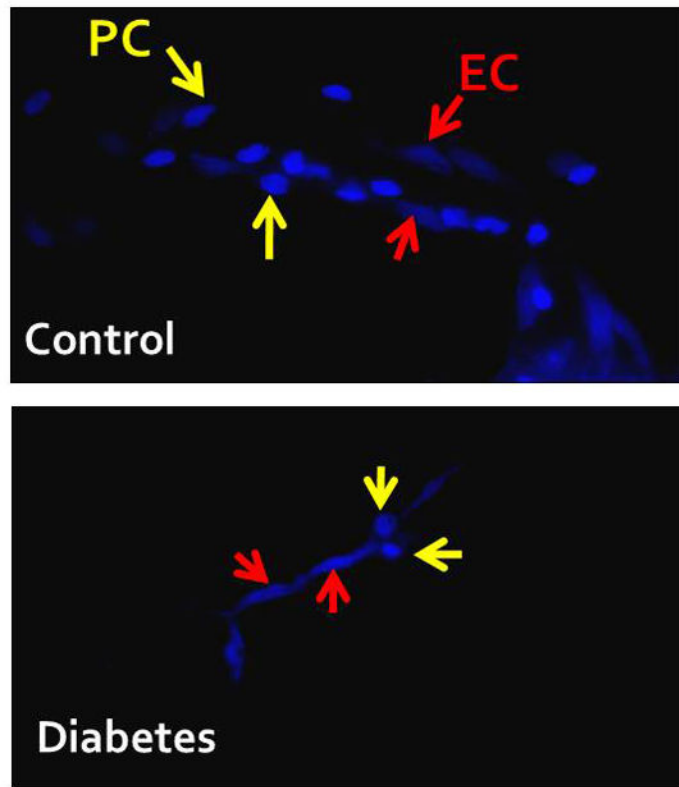


Figure 3. Diabetes reduces pericytes in the brain

Brain capillaries were isolated from control and diabetic Goto-Kakizaki rats 6–7 weeks after the onset of diabetes. Samples were allowed to adhere to cover slips and stained with nuclear stain DAPI. To determine the number of pericytes (PC), round nuclei were counted versus endothelial cells (EC) that have elongated nuclei.

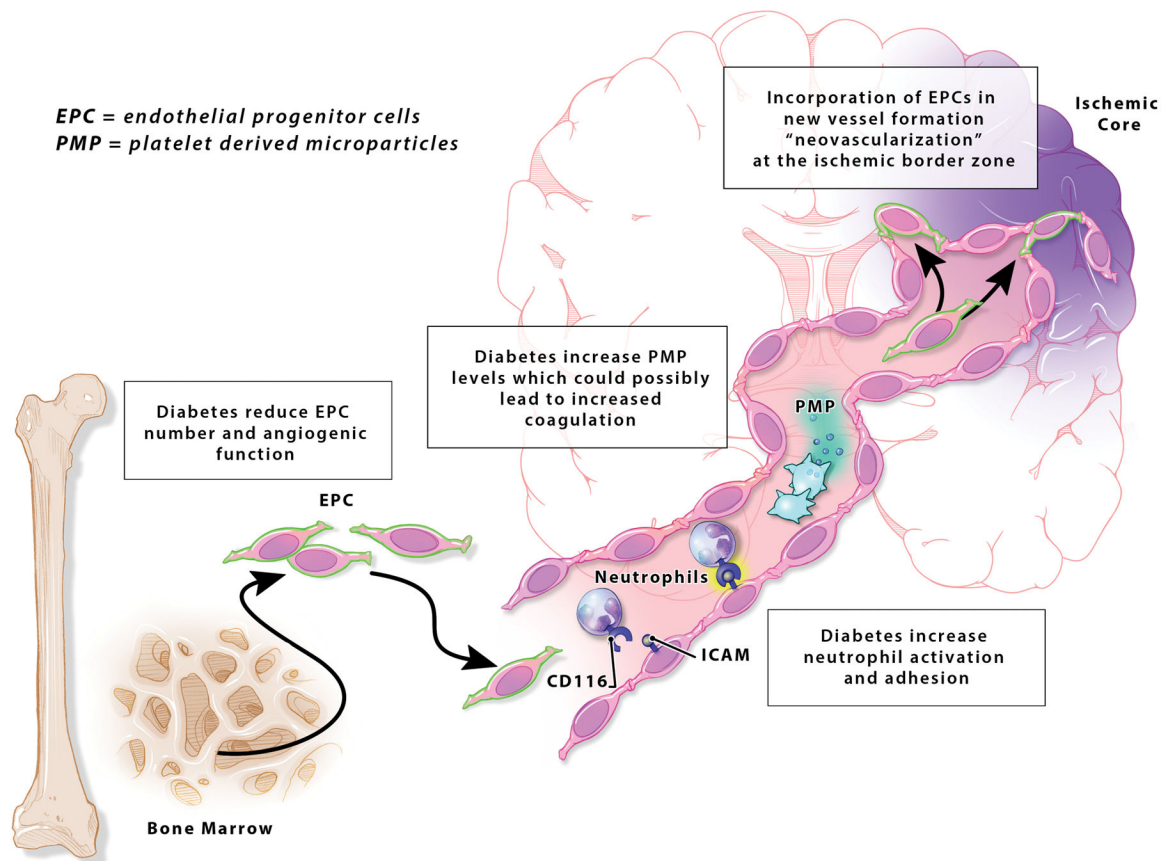


Figure 4. Effect of diabetes on systemic cells' functions after stroke

Bone marrow endothelial progenitor cells (EPC) produce angiogenic mediators and are incorporated in the newly formed vessels at the ischemic border zone after stroke. Diabetes reduces EPC count and angiogenic response. Diabetes increases neutrophil recruitment, activation and adhesion in the diabetic brain after stroke leading to increased inflammation. Diabetics have elevated levels of platelet-derived microparticles (PMP) putting them at risk of increased coagulation after ischemic stroke.

Table 1

Articles studying circulating cells response in diabetic stroke models

Author	Animal model	Stroke model	Circulating cells response	Stroke outcome	Follow up time
(Ritter et al., 2011)	Zucker Diabetic Fatty (ZDF) rats – type 2 diabetic	Intraluminal filament model - 2 h MCAO	Increased neutrophil activation, adhesion and decreased shear rate. Increased expression of CD11b and sICAM	Worse infarct size and neurologic function.	4 h after reperfusion
(Chen et al., 2011a)	Type 2 diabetic db/db mice	Intraluminal filament model - Permanent MCAO	In diabetic animals, BM-derived EPCs were reduced and dysfunctional as measured by reduced tube formation ability in vitro. The levels of circulating EPC-MPs and EMPs were increased in db/db mice.	Larger infarct volume	48 h after MCAO
(Kim et al., 2014)	Diet induced type 2 diabetes in mice	Intraluminal filament model - 30 min MCAO	Increased MCP-1 expression in peritoneal immune cells. Blunted inflammatory response in diabetic brain manifested by decreased MCP-1 RNA. Attenuated LPS-stimulated inflammatory response in macrophages.	Exacerbate d ischemic brain injury and infarct.	3 days post ischemia

MCAO: middle cerebral artery occlusion; CD11b: cluster of differentiation molecule b; sICAM: soluble intercellular adhesion molecule; BM: bone marrow; EPCs: endothelial progenitor cells; EPC-MPs: endothelial progenitor cell-derived microparticles; MCP-1: monocyte chemoattractant protein 1; LPS: lipopolysaccharide.

Table 2

Interventional studies in diabetic stroke using circulating cells

Author	Animal model	Stroke model	Treatment	Stroke outcome	Follow up time
(Chen et al., 2011a)	Type 2 diabetic db/db mice	Intraluminal filament model - Permanent MCAO	EPCs preincubated with <i>db/+</i> MPs or <i>db/db</i> MPs were infused 2 h after MCAO	infusion of EPCs preincubated with <i>db/+</i> MPs increased the level of cEPCs and reduced ischemic damage, and these beneficial effects were reduced or lost in EPCs preincubated with <i>db/db</i> MPs.	48 h
(Chen et al., 2011b)	STZ induced type 1 diabetic Wistar rats	Intraluminal filament model - 2 h MCAO	IV injection of BMSC at 24 h after MCAO	Increased mortality, BBB leakage and brain hemorrhage	14 days after stroke
(Chen et al., 2012)	Type 2 diabetic db/db mice	Intraluminal filament model - Permanent MCAO	Infusion of Ad-CXCR4 primed EPCs	Increased level of circulating EPCs and CXCR4 expression in the brain. Decreased infarct volume and neurological deficit with treatment	7 days after stroke
(Yan et al., 2013)	STZ induced type 1 diabetic Wistar rats	Intraluminal filament model - 2 h MCAO	IV injection of BMSC at 24 h after MCAO and Niaspan 40 mg/kg orally daily for 14 days	Combination treatment decreased BBB leakage but did not improve infarct volume or functional outcome.	14 days after stroke
(Ye et al., 2013)	STZ induced type 1 diabetic Wistar rats	Intraluminal filament model - 2 h MCAO	IV injection of BMSC at 24 h after MCAO and Niaspan 40 mg/kg orally daily for 14 days	Combination treatment increased white matter remodeling and synaptic plasticity.	14 days after stroke
(Yan et al., 2014)	STZ induced type 1 diabetic Wistar rats	Intraluminal filament model - 2 h MCAO	IV injection of HUCBCs at 24 h post-MCAO	Treatment improved functional outcome and enhanced vascular and white matter remodeling without affecting the infarct volume.	14 days after stroke
(Bai et al., 2014)	Type 2 diabetic db/db mice	Photothrombotic stroke - Permanent MCAO	Infusion of BM derived EPCs 24 h after stroke.	Less pronounced angiogenic response and delayed behavioral recovery compared to wild-type controls.	21 days after stroke

MCAO: middle cerebral artery occlusion; STZ: streptozotocin; EPCs: endothelial progenitor cells; MPs: microparticles; BMSC: bone marrow stromal cells; Ad: adenovirus; CXCR4: chemokine receptor type 4; HUCBCs: human umbilical cord blood cells.