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Emerging Therapy for Diabetic Neuropathy: Cell Therapy Targeting Vessels and Nerves

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Abstract

Diabetic neuropathy (DN), the most common complication of diabetes, frequently leads to foot ulcers and may progress to limb amputations. Despite continuous increase in incidence, there is no clinical therapy to effectively treat DN. Pathogenetically, DN is characterized by reduced vascularity in peripheral nerves and deficiency in angiogenic and neurotrophic factors. We will briefly review the pathogenetic mechanism of DN and address the effects and the mechanisms of cell therapies for DN. To reverse the changes of DN, studies have attempted to deliver neurotrophic or angiogenic factors for treatment in the form of protein or gene therapy; however, the effects turned out to be very modest if not ineffective. Recent studies have demonstrated that bone marrow (BM)-derived cells such as mononuclear cells or endothelial progenitor cells (EPCs) can effectively treat various cardiovascular diseases through their paracrine effects. As BM-derived cells include multiple angiogenic and neurotrophic cytokines, these cells were used for treating experimental DN and found to reverse manifestations of DN. Particularly, EPCs were shown to exert favorable therapeutic effects through enhanced neural neovascularization and neuro-protective effects. These findings clearly indicate that DN is a complex disorder with pathogenetic involvement of both vascular and neural components. Studies have shown that cell therapies targeting both vascular and neural elements are shown to be advantageous in treating DN.

Keywords

Angiogenic factors; cell therapy; diabetes mellitus; diabetic neuropathy; neurotrophic factors; nerves; vasa nervorum

Introduction

Diabetic neuropathy (DN) is a peripheral nervous system disorder and the most common complication of diabetes mellitus. There are 23.6 million children and adults in the United

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States (around 8% of the population) who have diabetes. DN affects up to 60% of long-standing diabetic patients [1]. Patients afflicted with DN experience decreased quality of life due to chronic pain, loss of sensation in the feet and other areas of the body, and chronic wounds partly caused by impaired pain response [2]. Autonomic nerve dysfunction also contributes to deterioration of the quality of life in diabetic patients [3].

DN can affect sensory, motor and autonomic nerve fibers in any part of the body. The nerves of the lower extremities usually become symptomatic first because they have the longest nerve fibers. There are several distinct syndromes based on the organ systems and types of nerves affected. A patient may have exclusively sensorimotor or autonomic neuropathy or a combination of both. Symptoms develop gradually over time and correlate with the degree of hyperglycemia.

Currently, there are no clinically validated, curative treatments for DN. Optimization of glucose control and foot care may halt disease progression but they cannot reverse nerve damages which often lead to debilitating secondary complications over time. Symptomatic treatment with pain medications is only partially effective and wounds are difficult to treat. Moreover, deficiency of neurotrophic factors has been regarded as one of the likely mechanisms underlying DN [1, 4]. In a clinical trial, a single treatment of injected neurotrophic cytokines was ineffective for treating DN [5]. Since DN lack both angiogenic and neurotrophic factors, cell therapy has recently emerged as an attractive therapeutic strategy in DN.

Pathogenetic Mechanisms Underlying DN

Although DN has been widely explored over the past 20 years [6] and its pathology has been well established, the pathogenesis remains unclear [7]. Pathological findings reported in diabetic patients include axonal atrophy, demyelination, nerve fiber loss, and blunted regeneration of nerves [1, 6]. The pathogenesis of DN is multi-factorial, involving both metabolic and vascular components [8, 9]. On a molecular level, the primary risk factor is hyperglycemia, which is associated with five pathways: the polyol pathway [10]; the advanced glycation end-product pathway [11, 12]; the protein kinase C pathway [13]; the poly ADP-ribose polymerase (PARP) pathway [14, 15]; and the hexosamine pathway [16]. The five pathways contribute to the production of oxidative stress. Accumulation of reactive oxygen species (ROS) increases lipid, DNA, and protein peroxidation, induces cellular apoptosis and, and reduces nerve blood flow [17, 18]. Increased oxidative stress leads to activation of the PARP pathway [19], which regulates the gene expressions involved in inflammatory reactions and neuronal dysfunction. Several studies suggest that oxidative stress and these five pathways are interdependent and central to the pathogenesis of neurovascular dysfunction [20-22].

On a cellular level, hyperglycemia affects sensory, motor, and autonomic neurons by activating the five pathways [1, 23]. Moreover, the induction of microvascular ischemia by reducing blood flow results in nerve dysfunction. ROS and reactive nitrogen species are associated with microvascular complications of diabetes [24-28]. ROS also contributes to impaired vasodilation of epineural blood vessels, resulting in ischemia to the neural tissue [29-31]. Oxidative stress leads to deterioration of Schwann cells, which play a key role as a

provider of insulation for neurons, immunologic perineurial blood-nerve-barrier, and effector of nerve regeneration. Such dysfunction *via* oxidative stress contributes to the phenotype of DN. Thus, antioxidants have become the therapeutic targets in DN studies. However, only a few studies have suggested that antioxidants can prevent or reverse hyperglycemia-induced nerve dysfunction in experimental DN models [32, 33].

Deficiency of Neurotrophic Factors and Vascular Supply as a Cause of DN

In addition to the classical pathogenesis mentioned above, studies have elucidated the major pathophysiologic role of neurotrophic factors and vascular supply in DN. The loss of neurotrophic support and ischemic hypoxia are widely considered to represent the two downstream consequences of the cellular mechanisms described above.

Changes in Growth Factors as a Cause for DN—Many representative growth factors have dual effects of being both neurotrophic and angiogenic [34]. Some examples are vascular endothelial growth factor (VEGF) [35], insulin-like growth factor (IGF) [36-38], nerve growth factor (NGF) [39-41], brain-derived neurotrophic factor (BDNF) [42, 43], and fibroblast growth factor-2 (FGF2) [44, 45]. Recently, the term angioneurin was coined to refer to a growth factor which have both angiogenic and direct neurotrophic effects [46]. The levels of these angioneurins were decreased in diabetic animals and were associated with neural function [47, 48].

VEGF, a major angiogenic factor, is a potent selective mitogenic cytokine for endothelial cells and its expression can be induced by hypoxia through hypoxia-inducible factor-1 [49]. In ischemic tissues, VEGF induces angiogenesis by stimulating the proliferation and migration of endothelial cells [50], leading to the improvement of tissue ischemia. VEGF also enhances Schwann cell migration [51] and proliferation, promotes axonal outgrowth and survival of both the neurons and Schwann cells of superior cervical ganglia and dorsal root ganglia [52]. IGFs induce vessel remodeling [38] and also have neurotrophic effect. IGFs have been shown to promote neurite outgrowth of neuroblastoma cells [53, 54] and accelerate regeneration of sensory [55] and motor nerves [56]. IGF1 is widely expressed in craniofacial sensory ganglia, sciatic nerve, spinal cord, sensory dorsal root ganglia and brain. IGF2 is expressed in the brain, vascular structures of the nervous system, and motor neurons. In neuronal cell bodies, axons and nerve terminals, IGF receptors (IGF1R and IGF2R) are present and IGF-1 expression is reduced in streptozotocin-induced diabetic rats compared to non-diabetic controls. mRNA and protein expression of both IGF1 and IGF2 is decreased in the nerves of streptozotocin-induced diabetic rats and there is also decrease in the mRNA and protein expression level of IGF1R in the superior cervical ganglia of streptozotocin-diabetic rats [57]. Also, Schwann cell mitogenesis and myelination are stimulated by IGF1 [58]. These effects may be important for inter-neuronal signaling and peripheral nervous system function. Sonic hedgehog (SHh) modulates patterning and development of embryonic nervous system. In diabetic animal, SHh mRNA levels are significantly decreased in peripheral nerves. In addition, overexpression of SHh improves blood flow to ischemic nerve and ameliorates nerve function [59]. NGF, a well-known neurotrophic factor, was initially identified as a molecule that promoted survival and differentiation of sensory and sympathetic neurons. Now, NGF has been shown to subserve

neuroprotective and repair functions [60]. NGF is synthesized by Schwann cells and target cells of sensory and sympathetic neurons such as epithelial cells, smooth muscle cells, and fibroblasts [61]. NGF homozygous knockout mice do not develop proper sympathetic neurons or small neural crest-derived sensory neurons [62]. In addition to these neurotrophic effects, NGF directly induces angiogenesis [40].

Vascular Deficiency as a Cause for DN—Maintaining adequate blood supply to nerves is crucial in maintaining nerve structure and function. Deficiencies in the blood supply to neural tissues through vasa nervorum, blood vessel within peripheral nerves largely contribute to pathogenetic mechanism of DN [63]. Several mechanisms on vascular structural changes in ischemia on diabetic nerve have been proposed. The most common abnormality in the structure of diabetic vasa nervora is the thickening of basement membrane [64-69], which is highly correlated with neuropathic severity [64, 70, 71]. In addition, decrease in nerve conduction velocity (NCV) in diabetic rats is preceded by impaired vasodilation in epineurial arterioles, which is partly mediated by ROS production [29-31]. In contrast to constricted epineurial arteriole, endoneurial capillaries appear to have a variable patency. Luminal areas of endoneurial capillaries were increased in rodent [72-74] and feline [75] models of DN, whereas those in human showed mixed results of being increased [64, 66, 76], unchanged [65, 69, 77], or decreased [67, 68, 70, 78, 79].

Also, mixed reports on blood vessel number or density in the nerves of diabetic subjects are apparently conflicting. In animal models of diabetes, endoneurial capillary density was reported to be increased [74, 80], unaltered [81], or decreased [47, 48, 82]. In human, the endoneurial capillary density was reported to be higher in early diabetic patients than healthy subjects [77]. Conversely, the endoneurial capillary density in diabetic patients with established neuropathy, showed no significant difference to that of healthy subjects [64, 65, 70]. However, recent studies have showed decreased functional capillary density using lectin perfusion as a method for measuring capillary density [47, 48, 82]. This discrepancy in the number of endoneurial capillaries appears to result from what methods or markers were used to examine capillaries. Studies altogether suggest that the number of capillary increases as compensatory response to ischemia in early diabetic condition and decreases, particularly the functional capillaries, due to impaired neovascularization under prolonged diabetic condition [72, 83, 84].

Despite some controversy on the structural aspects, it appears clear that DN is accompanied by ischemia and hypoxia of microcirculatory nutrient vessels in nerves [22, 63]. Because microcirculation is regulated by humoral, endothelial and neural factors, a vicious pathogenic cycle may develop: microcirculatory dysfunction results in peripheral nerve dysfunction which in turn results in abnormal regulation of the microcirculation leading to further nerve dysfunction. The reduction in endoneurial blood flow has been shown to be ameliorated by treatment with various vasodilatory agents, such as prostaglandin E1 analogues, alpha-adrenergic receptor blockers, angiotensin-converting enzyme inhibitors, angiotensin II receptor antagonists and endothelin receptor antagonists [73, 85-89] in animal models of diabetes.

Further evidence for the impaired vascularization and ischemia in DN are provided by the finding that there is a decrease in factors that promote or maintain blood vessel formation in DN (such as VEGFs, angiopoietins, IGFs, NGF). These observations led to studies of local delivery of the angiogenic factors VEGF-A and VEGF-C into diabetic rats which showed increased vasa nervora density and NCVs [63]. Additionally, VEGFs have direct neurotrophic effects which may underlie improvement of NCVs. For instance, angiogenic medications such as statins have also been shown to improve nerve function in DN [90].

Potential Therapies for DN

Several possible therapies exist for the treatment of DN based on neurovascular pathogenesis. Among them are gene, protein, and cell therapies.

Gene and Protein Transfer—Growth factors are attractive therapeutic option for DN because they can promote neuron survival and functional integrity, as well as repair of damaged nerves. Some growth factors are angiogenic, and their therapeutic effects are mediated by blood vessel growth that supply nutrients and oxygen to nerves [91]. Other growth factors, such as neutrophin 3, are neurotrophic, and their therapeutic effects are *via* promoting neural regeneration and survival [92]. Growth factors, known as angioneurins (VEGF, FGF2, NGF, BDNF, IGF1), have both angiogenic and neurotrophic properties [46]. The power of these growth factors in the treatment of DN was shown by Schratzberger *et al.,* [51, 63]. They were the first to inject VEGF encoding plasmids into rat and rabbit models of diabetes. The VEGF-treated animals showed normalization in NCV, increase in angiogenesis of vasa nervora, and increase in nerve fiber density. When the plasmid VEGF was applied to human patients, mild, but statistically significant symptomatic improvement was observed in a randomized, double-blinded trial [93]. However, the authors also reported that VEGF therapy was associated with adverse side effects that did not reach statistical significance. As this study has a relatively small sample size, further study is required to conclusively determine the effects of plasmid VEGF therapy. Other growth factors, such as IGF1 and IGF2 have also been studied in animal models of DN and have shown protective effects against development of neuropathy independent of changes in blood glucose [37]. The neurotrophic factor, FGF2 promotes angiogenesis and neurogenesis [94]. Diabetic rats treated with recombinant FGF2 showed improved nerve blood flow, motor NCV and response to mechanical stimuli 30 day post-injection. These results suggest that FGF2 supplementation is a potential therapeutic target of DN [95].

However, most of human clinical trials employing growth factors for DN have not shown beneficial effects [96] except for VEGF [93]. This may reflect the complexities of DN extending to treatment in humans in addition to variables such as period, mode, and delivery dosage of treatment.

Cell Therapy—As mentioned, emerging evidence have indicated that angiogenic factors such as VEGF-A, VEGF-C, SHh, and statin restore microcirculation in the affected nerves accompanied by functional improvement [63, 82, 90]. On the other hand, lack of neurotrophic factors has been regarded as an important pathogenic mechanism of DN [1, 4]. Administration of neurotrophic factors such as NGF [97], IGF1 and IGF2 [36, 37], ciliary

neurotrophic factor (CNTF) [98], or glial cell line-derived neurotrophic factor (GDNF) [99] was shown to ameliorate DN in animal models.

These findings suggest that a therapeutic modality which can target both angiogenic and neurotrophic processes may have more value in treatment of DN. In this sense, cell therapy using stem or progenitor cells has advantages over single gene or protein therapy. Cell therapy can increase multiple angiogenic and neurotrophic factors and potentially supplement specific type of cells required for vascular or neuronal regeneration (Fig. 1). Currently, various bone marrow (BM) cells were shown to have favorable effects for treating DN. An advantage of using circulating or BM-derived cells is that they can be harvested from a patient's own peripheral blood or bone marrow, and re-introduced back to the patient [100, 101].

Therapeutic Potential of Bone Marrow Mononuclear Cells: BM-MNCs are derived from BM and isolated using density gradient centrifugation. BM-MNCs are heterogeneous cell population including lymphocytes, hematopoietic stem/progenitor cells, EPCs and MSCs. BM-MNCs have been shown to augment neovascularization by increasing a broad range of angiogenic factors, including FGF2, VEGF, and angiopoietin-1 in the tissue [102, 103]. In animal models, transplantation of BM-MNCs into ischemic limbs [103] and myocardium [102] increased neovascularization and collateral blood vessel formation. These effects of BM-MNCs have also been documented in patients with limb ischemia in randomized controlled trials [104]. BM-MNCs are easily isolated and do not have to be expanded by *ex vivo* culture. This ease of isolation makes BM-MNCs an attractive source of cells for therapeutic neovascularization.

Recent studies have shown favorable therapeutic effects of BM-MNCs on experimental DN. Hasegawa and colleagues showed that implantation of either peripheral blood (PB)-MNCs or BM-MNCs in a rat model of DN improved motor NCV and blood flow around the sciatic nerve, which is possibly mediated by VEGF secreted from MNCs. This study suggests that BM-MNCs are more effective than PB-MNCs as BM-MNCs include significantly more EPCs than PB-MNCs [81]. Recently Kim *et al*., reported that intramuscularly transplanted BM-MNCs preferentially localize to the nerves in diabetic rats, especially around vasa nervorum, and increase expression of various angiogenic and neurotrophic factors in the nerves [48]. The vascularity of these nerves improved (Fig. 2) and NCV levels were almost normalized [48]. These studies suggest that the vasa nervorum may play a pathogenic role in both the development and reversal of DN. This study further suggested that angiogenesis is the central mechanism of BM-MNC-induced neovascularization in experimental DN because, from their observation, BM-MNCs do not differentiate into, nor fuse with, endothelial cells in the nerves at a detectable level.

Therapeutic Potential of Endothelial Progenitor Cells and Postnatal Vasculogenesis: The development of vascular system consists of two processes: vasculogenesis and angiogenesis. Vasculogenesis refers to the *de novo* formation of blood vessels from EPCs or angioblasts that differentiate into endothelial cells [105], whereas angiogenesis is growth of pre-existing vasculature by sprouting of new capillaries through proliferation and migration of endothelial cells [106]. Until recently, vasculogenesis was thought to be restricted to

embryonic development, while angiogenesis was considered to be responsible for neovascularization in embryos and adults. This view was challenged with the discovery of BM-derived EPCs, which circulate in adult peripheral blood [107], home to ischemic tissue and incorporate into foci of neovascularization [108], leading to *de novo* blood vessel formation.

The identity of EPCs is complicated by the complexity of the definition and various methods to define the cells. It is now apparent that different subsets of peripheral or BM derived cells, including hematopoietic stem cells, monocytes and circulating endothelial cells, can differentiate into endothelial-like cells. BM-derived EPCs in the adult peripheral blood express a subset of hematopoietic stem cell markers [109, 110]. Specifically, CD34, CD133 and VEGF receptor-2 have been proposed as candidate markers for human EPCs [110-112]. However, there are no known specific markers to identify EPCs without cultivation. *Ex vivo* expanded human EPCs express various endothelial cells markers such as CD31, CD34, KDR, VE-cadherin, bind lectins, and incorporate Dil-acetylated low-density lipoprotein. The origin of EPCs is further obscured by the two distinctive types of EPCs arising from different culture methods [113]. “Early EPCs”, are mainly derived from mononuclear cells or monocytes and do not proliferate after a few weeks [114-116]. On the other hand, “Late EPCs” form colonies after more than two weeks in culture, have cobblestone morphology, and rapidly proliferate [114, 117]. The distinctive characteristics of these two types of EPCs are reinforced by the different expression of cell surface markers. Early EPCs express pan-leukocyte and monocytic/macrophage markers such as CD45, CD11b and CD14 while late EPCs do not. Early EPCs are also therapeutically effective *in vivo* while evidence for therapeutic efficacy of late EPCs are limited to date [114, 117].

Endothelial differentiation of EPCs and whether this differentiation plays a main role in the therapeutic benefit of EPCs in recovering damaged tissue function is controversial. Several recent studies have demonstrated differentiation of EPCs into endothelial lineage cells with incorporation into blood vessels [118, 119]. However, other investigators claim that BM-derived cells including EPCs do not undergo endothelial differentiation nor incorporate into vessel walls [120, 121]. These discrepancies may be due to the difference in cell types, the use of different animal models or the rigor criteria to define endothelial differentiation.

One study showed that cord-blood derived EPCs were effective for treating DN [122]. This study claimed that mechanistically, the therapeutic effects are due to the increased differentiation of EPCs into endothelial cells in hindlimb muscles, which then led to an increase in sciatic nerve blood flow. However, this study did not demonstrate the fate of the EPCs in tissues, nor did it address the mechanisms by which transplanted EPCs increase neovas-cularization in muscles or nerve. Given that most recent studies have argued against the endothelial differentiation of EPCs as a major mechanism for neovascularization, endothelial differentiation does not appear to underlie such magnitude of therapeutic effects toward DN [115, 123].

More recently, a study by the author's group reported that local transplantation of BM-derived EPCs improved various manifestations of experimental DN through dual angiogenic and neurotrophic effects on peripheral nerves (Fig. 1) [47]. This study uncovered some

important mechanistic insight into the role of EPCs on DN [47]. First, intramuscularly injected EPCs exert therapeutic effects through direct modulation of nerves, not through muscular neovascularization. Second, the therapeutic effects of EPCs are mainly mediated by humoral or paracrine factors released by EPCs, rather than the direct endothelial differentiation of EPCs. Third, the functional capillary (vasa nervora) density in the nerves was significantly increased by the EPC treatment. Fourth, Intramuscularly injected EPCs preferentially homed to sciatic nerves, characteristically localized in close proximity to vasa nervora, and differentiated into endothelial cells albeit infrequently [47]. A large number of engrafted EPCs survived in peripheral nerves for more than 12 weeks and induced prolonged expression of angiogenic and neurotrophic factors. Fifth, EPC transplantation increased proliferation and decreased apoptosis of endothelial and Schwann cells (Fig. 1).

The most notable finding was the direct effect of EPCs on peripheral nerves. The study was the first to demonstrate that EPC transplantation increases capillary density and blood flow in nerves, suggesting that EPCs induce neovascularization in nerves [47]. The differentiation of EPCs into endothelial cells, histologically confirmed as the colocalization of DiI-labeled transplanted cells with BS-1 lectin positive endothelial cells was infrequent, suggesting that angiogenesis could have played a more important role than vasculogenesis. This neural angiogenesis occurred through upregulation of various angiogenic factors in nerves after EPC transplantation. Various paracrine factors including VEGF-A [51, 63], FGF2 [124], BDNF [42], SHh [59, 82], and stromal cell derived factor (SDF)-1α [125, 126] were expressed in the peripheral nerves (Fig. 1). These factors have been shown to have synergistic effects on angiogenesis as well as neuro-protecting effects [63, 95, 127]. In fact, this study was the first to show clear dual angiogenic and neurotrophic effects of EPCs. This upregulation of various classes of biologically important factors may be one of the greatest benefits of stem cell therapy over any single protein or gene therapy, enabling the concerted efforts of multiple neuro-angiogenic cytokines necessary for neurovascular recovery.

Histologically, the author's study also uncovered novel engraftment and retention characteristics of BM-derived cells in tissues [47]. Following a series of reports on the short-term engraftment of any BM cells in a myocardial infarction model [128, 129], the prevailing notion was that adult stem/progenitor cells could not sustain their engraftment more than a few weeks. However, the study by Jeong *et al*., clearly rebutted this notion that BM-EPCs could survive more than 12 weeks in nerves [47]. The EPCs which were directly injected into the hindlimb muscles disappeared mostly in the muscles within 8 weeks; however the EPCs robustly survived for more than 12 weeks in the sciatic nerves. Interestingly, the study by Naruse *et al.*, showed that capillary density, which had decreased in hindlimb muscles of diabetic rats at 12 weeks of diabetes, was significantly increased after cord-blood EPC treatment [122]. However, this study suggested that blood flow and capillary density in hindlimb were not significantly changed after EPC treatment. This long-lasting cell retention is compatible with the observation that EPCs homed to peripheral nerves far more preferentially than to muscles. This scale of close interaction between any BM cells and steady-state tissues was not previously reported with or without diabetes. The long-term retention of EPCs into the nerves in diabetic mice was not expected when we started this project. Together, our studies with EPCs or BM-MNCs strongly argue that the

engraftment characteristics of BM cells depends more on the recipient environment than on the transplanted cells themselves [47, 48]. Another intriguing finding was that the engrafted EPCs were localized in close proximity to the vasa nervora. Such a large magnitude of tropism of BM-derived cells to blood vessels has not been reported in any other tissues. This also clearly supports that despite the controversy of EPCs on blood vessel forming capability in certain models like myocardial infarction [123, 128, 130], it is evident that EPCs can play an important role in vessel homeostasis [123, 131-134]. The distinct properties of BM-derived EPCs such as peripheral neurotropism, sustained engraftment, and vascular localization of EPCs induced robust and prolonged paracrine or humoral effects and reversal of various functional and pathologic features of DN [47, 48, 81, 122].

Perspective

DN is a progressive disease and its manifestations can take many years to develop. Cell therapy may not be a standard treatment option for all stages of DN because different stages of DN are marked by different structural or functional changes. At present, cell therapy may be applied to those patients who suffer from intractable symptoms, acute exacerbation, or combined diseases such as diabetic foot ulcers or critical limb ischemia.

Practically, as the safety of autologous BM-derived cells has been documented by a number of clinical trials [135], it is highly recommended to advance this strategy into a pilot clinical trial for those who are severely affected by DN. Particularly, EPCs will be effective in treating DN when combined with diabetic wounds or peripheral vascular obstruction as the therapeutic effects were already shown in these conditions. However, there are a few remaining concerns in cell therapy strategy. First, the effectiveness of the patient's own cells needs to be evaluated considering the possibility that BM cells derived from diabetic subjects may be impaired in therapeutic potential. Experiments using the autologous cells derived from diabetic subjects are necessary to address these concerns. However, although the efficacy of autologous diabetic cells is less potent, there may be still ways to overcome these defects to a certain extent. One strategy is to enhance their angiogenic and neurotrophic effects by culturing cells and activating necessary pathways with small molecules or growth factors. Second, the long-term effects of cell therapy need to be tested. Given that DN is a disease progressing over a long time, a single injection of cells may not be enough to maintain the nerve function over a long period of time. There are a few approaches to take in this context. One approach is to implant cells repeatedly to maintain their effects. At present, the duration of the beneficial effects of cell therapy in DN is unknown and a critical issue that requires further investigation. In many cases, the first manifestation of DN is a diabetic foot or ulcer which sometimes requires an amputation and a long-term care, which significantly reduces patients' quality of life. Cell therapy in this case can be very critical to rescue further tissue loss.

Cardiovascular autonomic neuropathy (CAN) is associated with increased risk of cardiovascular morbidity and mortality in diabetic patients [136, 137]. Although CAN is one of the most frequently studied complications of diabetes [138] and cell therapy has been reported to be effective in improving ischemic cardiovascular disease [132] and peripheral

neuropathy, cell therapy has yet been evaluated in either animal models or human patients with CAN. Future studies are required to determine the effects of cell therapy in CAN.

Future directions of cell therapy for DN will take steps toward enhancing the potency of candidate cells, using both gene and cell therapy, and working with combination of various cell types such as those derived from induced pluripotent stem (iPS) cells Once generated, iPS cells can offer a plentiful and renewal source of cells that can be induced to differentiate into cells of interest [139]. Conclusively, cell therapy may become an innovative alternative therapeutic option for treating advanced DN. However, further research is necessary to overcome some limitations and possible adverse effects of cell therapy.

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Abbreviations

BDNF	Brain-derived neurotrophic factor
BM	Bone marrow
DN	Diabetic neuropathy
EPC	Endothelial progenitor cell
FGF	Fibroblast growth factor
IGF	Insulin-like growth factor
MNC	Mononuclear cell
MSC	Mesenchymal stem cell
NCV	Nerve conduction velocity
NGF	Nerve growth factor NGF
PARP	Poly DP-ribose polymerase
PB	Peripheral blood
ROS	Reactive oxygen species
SHh	Sonic hedgehog
VEGF	Vascular endothelial growth factor

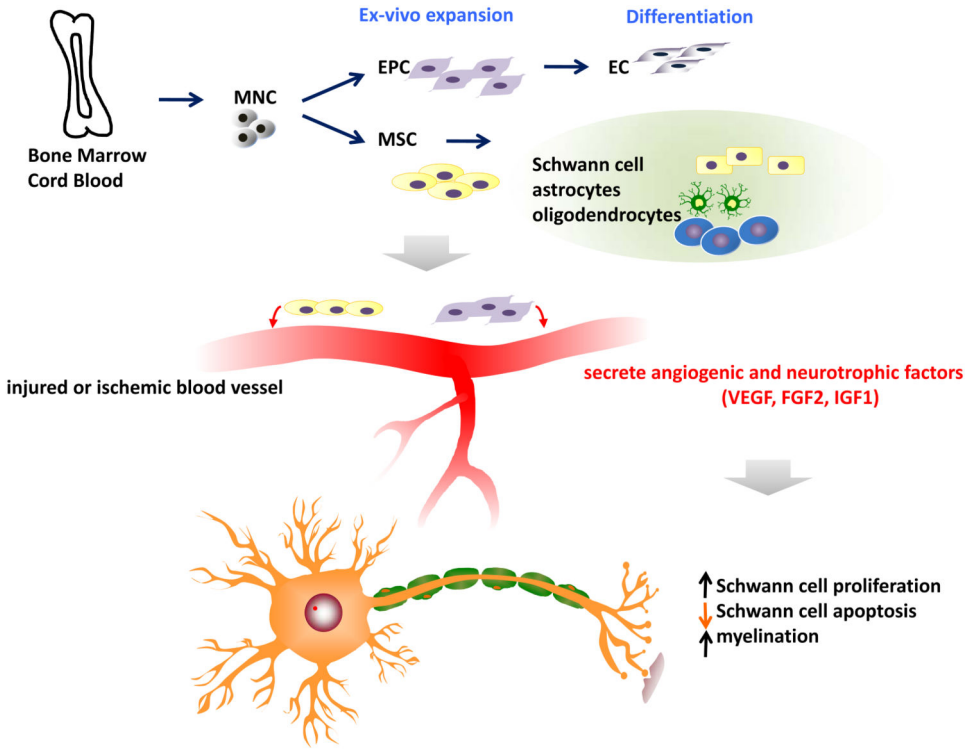

Fig. 1. Mechanism of action of various bone marrow-derived cells

Endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) can be cultured from mononuclear cells (MNCs). EPCs can differentiate into ECs and MSCs can give rise to Schwann cells, astrocytes, and oligodendrocytes. When transplanted into diabetic neuropathy animals with injured or ischemic blood vessels, the EPCs and MSCs secret angiogenic and neurotrophic factors including VEGF, FGF2, and IGF1, leading to increase in Schwann cell proliferation and myelination and decrease in Schwann cell apoptosis.

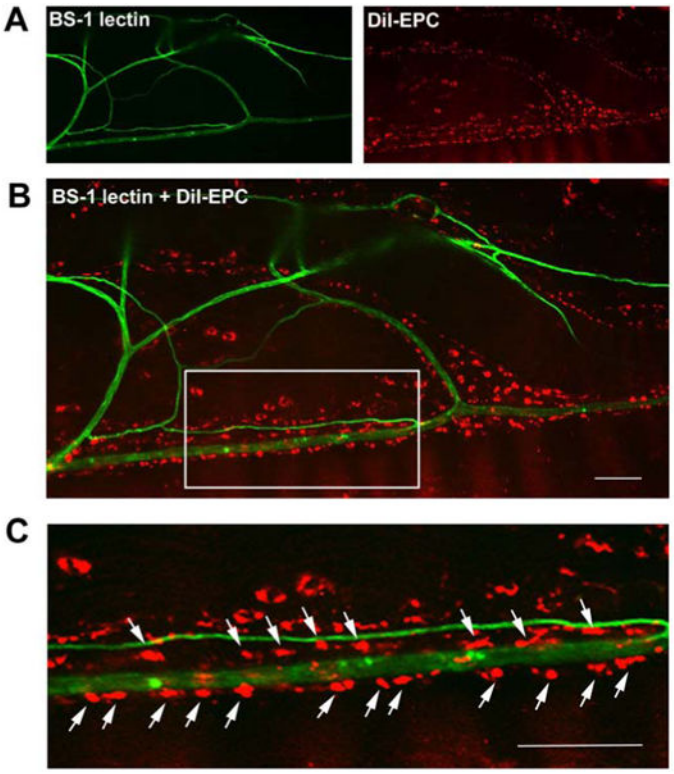

Fig. 2. Localization of transplanted EPCs along the vasa nervorum and transdifferentiation of EPCs into endothelial cells in nerves

EPCs were isolated from mouse bone marrow and labeled with a red fluorescent dye, CM-DiI. Streptozotocin-induced diabetic mice were injected with the CM-DiI-labeled EPCs into the muscles along the course of the sciatic nerve. Eight weeks after the EPC transplantation, the mice hindlimbs were perfused with BM-1 lectin conjugated with FITC to visualize the blood vessels and the sciatic nerves were harvested. Whole mounted images of a sciatic nerve (**A-C**) demonstrated that engrafted EPCs (red) preferentially localized along the course of the vasa nervorum (green). Bars, 50 μm in B and C.