

Stem Cells for Neurovascular Repair in Stroke

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Abstract

Stem cells exert therapeutic effects against ischemic stroke via transplantation of exogenous stem cells or stimulation of endogenous stem cells within the neurogenic niches of subventricular zone and subgranular zone, or recruited from the bone marrow through peripheral circulation. In this paper, we review the different sources of stem cells that have been tested in animal models of stroke. In addition, we discuss specific mechanisms of action, in particular neurovascular repair by endothelial progenitor cells, as key translational research for advancing the clinical applications of stem cells for ischemic stroke.

Keywords: Cerebral ischemia; Cell-based therapies; Vasculature; Blood brain barrier; Endothelial cells

Stroke: A Significant Unmet Clinical Need

Stroke is the third leading cause of death and the leading cause of long-term disability in the United States [1]. In 2004, the direct and indirect costs of stroke in the United States were estimated to be \$53.6 billion [2]. The mean lifetime cost of ischemic stroke to a single patient in the United States is estimated at \$140,048; this includes inpatient care, rehabilitation, and follow-up care necessary for lasting deficits. Approximately 2 out of every 1000 adults will have their first stroke in any given year in the United States [3]. The numbers of affected individuals, the costs necessary to facilitate their care, and rehabilitation coupled with the lack of therapies indicate that stroke represents a current significant unmet medical need.

The current therapy for stroke is limited [4-7]. Other than one recombinant protein therapy directed at the dissolution of thrombi in affected blood vessels in adults following stroke, tumor plasminogen activator or tPA, no specific treatment is available for either focal cerebral ischemia or global ischemic event. A major limitation with tPA is its very narrow therapeutic window of 4.5 hours after stroke onset. Beyond this timing of administration, tPA presents with deleterious side effects, in particular bleeding and hemorrhagic transformation, which can exacerbate stroke injury and counteract the benefits provided by reperfusion of the occluded artery [8]. To circumvent this limited tPA time to treat patients, telemedicine has been set-up in rural areas lacking access to medical centers [9,10] in order to advance diagnosis of ischemic stroke and access to tPA within the limited therapeutic period. Unfortunately, such tele-stroke medicine remains in infancy with significant health disparity between rural and urban stroke care contributing to small population of stroke patients benefitting from tPA [11,12]. Investigations to small molecule therapies such as anti-platelet drugs, anticoagulants and statins acting as prophylactics have not produced consistent benefit following an acute attack, whereas neuroprotective compounds such as albumin and minocycline are recently being explored in clinical trials [13,14]. Because tPA is already an FDA-approved drug, finding strategies designed to extend its therapeutic window seems a highly logical lab-to-clinic translational route for introducing a novel therapy for stroke. Hence, a potent research strategy that dovetails on tPA's safety and efficacy profile, but also recognizes the drug's limitation and adverse effects, may reveal new avenues of treatment for stroke. To this end, we advance the approach that cell therapy can abrogate the blood brain barrier (BBB) breakdown associated with tPA especially when given beyond the 4.5

hours, and such BBB repair should extend tPA's therapeutic window, as well as directly benefit stroke in view of the BBB damage inherent in the disease itself.

This paper discusses the preclinical basis for testing stem cell therapy in stroke. We outline below the potential of cell-based therapy in circumventing the current limitations and deleterious side effects of tPA for treating stroke. Finally, we address the gap in knowledge concerning mechanisms underlying the therapeutic benefit of stem cells in stroke. Here, we highlight the underexplored concept of neurovascular repair as a major mode of action of cell therapy, and emphasize the major role of endothelial progenitor cells (EPC) as an effective cell source for transplantation. Our strategy is to exploit this neurovascular repair mechanism via EPC transplantation as a stand-alone or as an adjunct therapy for augmenting tPA treatment for stroke.

Stem Cell-based Therapy for Stroke

Several sources of stem cells have been demonstrated as safe and effective in animal models of stroke. Recently we reviewed various kinds of stem cell sources in detail [15]. In a historical order, the major types of cells transplanted in stroke include fetal-derived cells, neuroteratocarcinoma cells (NT2N), xenogenic pig-derived cells, embryonic stem (ES) cells, adult stem cells (bone marrow, human umbilical cord, placenta, amnion fluid, menstrual blood), and induced pluripotent stem cells (iPS). Due to ethical and logistical concerns, the use of adult stem cells has flourished over the last decade, which was further aided by a moratorium for using federal funds on ES research. Interestingly, the ongoing FDA-approved stem cell clinical trials in stroke use adult stem cells. For this section, we highlight the potential of adult bone marrow-derived endothelial progenitor cells in neurovascular repair for stroke.

Cell transplantation therapies and stem cell treatments have

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emerged as potential treatments for numerous diseases and medical conditions, including stroke. One approach using stem cells involved the direct transplantation of neural stem cells (NSCs) into the damaged region of the brain. NSCs transplanted following transient global ischemia differentiated into neurons and improved spatial recognition in rats [16]. Post-mitotic neuron-like cells (NT2N) cells, derived from a human embryonal carcinoma cell line, migrated over long distances after implantation into brains of immuno-competent newborn mice and differentiated into neuron- and oligodendrocyte-like cells [17]. NT2N cells promoted functional recovery following focal cerebral ischemia after direct transplantation [18]. Similarly, MHP36 cells, a stem cell line derived from mouse neuroepithelium, improved functional outcome in rats after global ischemia [19] and also following focal cerebral ischemia or stroke [20]. NCSs grafted into brain developed morphological and electrophysiological characteristics of neurons [21].

Other direct transplantation experiments in the brain have utilized cells derived from bone marrow. Bone marrow stromal cells (MSCs), when injected into the lateral ventricle of the brain, migrated and differentiated into astrocytes [22]. Fresh bone marrow transplanted directly into the ischemic boundary zone of rat brain improved functional recovery from middle cerebral artery occlusion [23]. Similarly, MSCs implanted into the striatum of mice after stroke, improved functional recovery [24]. MSCs differentiated into presumptive neurons in culture [25] and assumed functional neuronal characteristics in embryonic rats [26]. Intracerebral grafts of mouse bone marrow also facilitated restoration of cerebral blood flow and BBB after stroke in rats [27]. Indirect transplant methods, via intravenous or intra-arterial injection, also have been shown to afford positive effects. Following bone marrow transplantation with tagged donor cells, tagged bone marrow stem cells were shown to differentiate into microglia and astrocytic-like cells [28]. Intra-carotid administration of MSCs following middle cerebral artery occlusion in a rat model improved functional outcome [29]. Similarly, intravenous administration of umbilical cord blood cells ameliorated functional deficits after stroke in rats [30]. Rats, which had received tagged bone marrow cell transplantation, showed the tagged cells as putative neurons and endothelial cells following middle cerebral artery occlusion and reperfusion [31]. It has also been reported that intravenous administration of cord blood cells was more effective than intra-striatal administration in producing functional benefit following stroke in rats [32]. Intravenous administration of MSCs has also been found to induce angiogenesis in the ischemic boundary zone following stroke in rats [33].

Cell Replacement and By-stander Effects of Stem Cell Grafts

It is unclear what brings about the purported benefit from stem cell transplantation. One possibility is the transformation of the transplanted cells into neurons [34]. There appears to be a positive relationship between the degree of behavioral improvement and the number of transplanted cells that stain positive for neuron specific markers [16]. However, transplanted cells often do not develop normal processes, and thus the benefit may not be mediated only by neuronal circuitry [35].

A second hypothesis that is not mutually exclusive is that the transplanted cells may also assist via differentiation into neuroectodermal derived cell types other than neurons. Marrow stromal cells migrate and transform into astrocytes [22]. Hematopoietic cells can differentiate into microglia and macroglia [28]. Bone marrow

derived stem cells may also assist in blood vessel regeneration following brain tissue damage in several ways. The stromal cell derived factor-1 (SDF-1)/CXCR4 system assists in integration of cells into injured tissue by promoting the adhesion of CXCR4-positive cells onto vascular endothelium [36]. SDF-1 also augments vasculogenesis and neo-vasculogenesis of ischemic tissue by recruitment of endothelial progenitor cells [37]. Bone marrow is a source of these endothelial progenitors [38]. Adult bone marrow-derived cells have been shown to participate in angiogenesis by the formation of periendothelial vascular cells [39]. Intravenous administration of MSCs induced angiogenesis in the ischemic boundary zone after stroke [33]. We also observed that crude bone marrow is a source of endothelial cells after experimental stroke [31].

Trophic factors produced by the transplanted cells could be a factor. Via this mechanism, bone marrow grafts may assist in restoring brain blood flow and also repairing the BBB [27]. Trophic factors from marrow stromal cells may play a role in brain repair itself. Recent evidence suggests that intravenous administration of MSCs increases the expression of nerve growth factor and brain-derived neurotrophic factor following traumatic brain injury [40]. Understanding the exact mechanism(s) responsible for the therapeutic benefit seen following stem cell transplantation in the CNS is now at a critical junction in view of the planned FDA allowance for limited clinical trials of bone marrow-derived multipotent adult progenitor cells in acute ischemic stroke [41].

In accordance with the STAIR (Stroke Therapy Academic Industry Roundtable) and STEPS (Stem cell Therapeutics as an Emerging Paradigm for Stroke) criteria, investigations of the mechanism of action mediating experimental therapeutics in stroke are vital for extending their potential clinical utility [42,43].

An Underexplored Stroke Therapeutic Target: BBB repair

A closely associated cell death cascade involved in stroke pathogenesis is impairment of the BBB, which further exacerbates brain damage. The central nervous system (CNS) is an immunologically privileged zone, protected from entry of immune cells and serum proteins by the BBB (as well as by the blood-spinal cord barrier and blood-cerebrospinal fluid barrier, but we will focus here on BBB). These CNS barriers control cerebral/spinal cord homeostasis by selective transport of molecules and cells [38-40,44,45]. This control is possible due to the unique structure of the microvasculature – in particular capillaries formed by endothelial cells which are connected via adherens and tight junctions [46-48]. Functional integrity of all BBB elements is critical for protection of the CNS from harmful blood substances. Impairment of this cellular machinery may cause BBB breakdown, leading to edema in many cases of brain diseases or injuries, including stroke. Degradation of the extracellular matrix may be concomitant with BBB disruption and tissue softening, leading to more pronounced brain swelling and to severe cerebral edema in stroke patients [49] and other brain disorders such as Alzheimer's disease [50] and multiple sclerosis [51,52]. Examination of BBB status in stroke reveals evidence of the barrier's altered permeability. Whereas the first phase of stroke is characterized by a surge in tissue Na⁺ and water content concomitant with an increased pinocytosis and Na⁺, K⁺ ATPase activity across the endothelium, the second stage of stroke ensues with BBB breakdown that is associated with infarction of both the parenchyma and the vasculature itself [53]. At this second stage, tissue Na⁺ level still remains, but the extravasation of serum proteases

stands as a likely exacerbating factor [54]. Accumulating evidence implicates serum proteases in degradation of the extracellular matrix metalloproteinases (MMPs), which in turn aggravate BBB disruption and softening of the tissue, eventually manifesting into a well-defined form of brain swelling [53-55]. Part of the reason for the tPA's limited time window is that the surge in production of free radicals associated with delayed reperfusion brings a second wave of oxidative and nitrative stress that increases the risk of brain hemorrhage and edema [56]. With delayed reperfusion, there is a surge in production of superoxide, NO, and peroxynitrate. Formation of these radicals in the vicinity of blood vessels plays an important role in reperfusion-induced injury. These radicals activate MMPs, which degrade collagen and laminin in the basal lamina, disrupting the integrity of the basement membrane and increasing BBB permeability. Oxidative and nitrative stress also triggers recruitment and migration of neutrophils and other leukocytes to the cerebral vasculature, which release enzymes that further increase basal lamina degradation and vascular permeability. These BBB pathological events can lead to parenchymal hemorrhage, vasogenic brain edema, and neutrophil infiltration into the brain [57]. In the clinic, significant brain edema, such as that seen in malignant MCA infarction, develops in a delayed fashion after large hemispheric strokes and accounts for a high mortality rate (80% in the case of malignant MCA infarction) [58]. The primary BBB function is controlling CNS homeostasis by selective transport. Substances with molecular weights higher than 400 Da generally cannot cross the BBB by free diffusion. Some molecules cross the barriers via endothelial carrier-mediated or receptor-mediated transporters, see review [38,39,44,59]. It is possible that barrier disruption or dysfunction occurs in stroke, altering CNS homeostasis and allowing entry of harmful molecules from the periphery to the brain [60-62]. Among these injurious molecules are immune/ inflammatory factors, such as monocyte/macrophage cells, activated microglia, and reactive astrocytes possibly secreting pro-inflammatory cytokines, which have been detected in stroke patients and animal models [63-65]. Although additional studies are warranted to confirm the BBB status in stroke patients, the above results taken together imply that BBB dysfunction may contribute to stroke pathology. Thus, there could be an impaired endothelium-mediated mechanism in stroke leading to barrier dysfunction.

EPC Therapy for BBB Repair in Stroke

Endothelial progenitor cells (EPCs), initially described by Asahara et al. [66] are immature endothelial cells that circulate in peripheral blood. In their pioneering study, transplanted EPCs, isolated from human blood, were found in the endothelium of newly formed vessels in ischemic regions, indicating that a discrete cell population within the human blood participates in the formation of new vessels after ischemia. Griese et al. [67] also found that grafted EPCs populated the endothelium in animals with experimentally induced endothelial damage, further advancing the notion that EPCs contribute to the repair of damaged endothelium. The dogma that existed until recently is that neovascularization, or formation of new blood vessels, results exclusively from proliferation and migration of pre-existing endothelial cells, a process referred as to angiogenesis [68]. Furthermore, vasculogenesis or vascularization, defined as *in situ* differentiation of vascular endothelial cells from endothelial precursor cells, was thought to occur only in the embryo during vascular development. However, recent evidence has now established that circulating bone marrow-derived EPCs are capable of homing to neovascularization sites, proliferating, and differentiating into endothelial cells [69,70]. EPCs have been identified mainly in the mononuclear cell fraction of peripheral blood, leukapheresis products, and in umbilical cord

blood [66,71], but can also be harvested from bone marrow. Over the last few years, EPCs have been studied as biomarkers to assess the risk of cardiovascular disease in human subjects. For example, a low EPC count predicts severe functional impairments in several cardiovascular pathologies such as diabetes [72], hypertension [73,74], scleroderma [75,76], aging [74,77], cigarettes smoking [74,78,79], and coronary artery disease [46]. In addition, EPCs have been examined as potent donor graft cells for transplantation therapy.

Transplantation of EPCs into ischemic tissues has emerged as a promising approach in the treatment of diseases with blood vessels disorders [80-82]. In mouse models of ischemic injury, EPCs injection led to improved neovascularization in hind limb ischemia [80-82]. Based largely on these laboratory findings suggesting angiogenic and vasculogenic potential of EPCs, clinical studies have been initiated to reveal whether patients with lower EPC numbers are at higher risk for atherosclerotic events, and whether patients with ischemic events may benefit from EPC administration [83].

Clinical studies to date suggest the therapeutic potential of EPC transplantation, although this assumption should be approached with much caution due to being open label trials, observational and/or anecdotal accounts, and limited number of patients. *Ex vivo* expanded EPCs, isolated from peripheral blood mononuclear cells, can incorporate into the foci of myocardial neovascularization [84,85], and intracoronary infusion of peripheral blood or bone marrow-derived progenitors in patients with acute myocardial infarction was associated with significant benefits in post-infarction remodeling [86-93]. Still in observational studies in patients with myocardial infarction, higher numbers of EPC correlate with better prognosis, more myocardial salvage [94], viability and perfusion [95], and more collaterals in the ischemic zone [96]. Randomized clinical trials on autologous bone marrow-derived cells are mixed; whereas transplanted coronary artery disease patients display improved left ventricular function at least in the short term [97], transplanted patients with chronic ischemic heart failure exhibit modest to no effects on change in left ventricular function [98].

Similar randomized trials of autologous bone marrow-derived cells have been carried out in patients with peripheral artery disease and showed improved endothelium-dependent vasodilation [99], ankle brachial index, rest pain, and pain-free walking time [100], but the degree of functional recovery was not as robust as seen in animal models. Clearly, these results are obtained from autologous bone marrow-derived cells, which are heterogenous with scarce number of EPCs, thus may not closely approximate EPC endpoints. For clinical application of EPCs in neurovascular disease, the available studies are much more limited with only 3 observational studies in patients with stroke. In 25 patients with an ischemic stroke, CD34+ cells peaked 7 days after stroke but generally reverted to baseline after 30 days [101]. Interestingly, higher CD34+ cell levels at 30 days related to higher numbers of infarcts on magnetic resonance imaging and also to cerebrovascular function as measured with positron emission tomography scanning (cerebral metabolic rate of oxygen, and cerebral blood flow). On the other hand, decreased numbers of clusters of rapidly adhering cells were seen after stroke and in "stable cerebrovascular disease," compared to controls free of vascular disease [102]. Higher age and the presence of cerebrovascular disease in general independently related to lower EPC numbers. The discrepancies in the results of these studies may be due to mismatched controls for age of patients and/or the lack of methodological design for testing specific hypotheses on the causal role of EPC in cerebrovascular disease [102]. Although the primary mitigating mechanisms underlying stroke pathogenesis and

its abrogation by cell therapy are still uncertain, there is substantial evidence implicating immunological attack upon the brain and/or its vasculature; widespread inflammatory reactions in stroke may trigger a cascade of events which alter the integrity of the BBB, resulting in migration of leukocytes into the CNS. Leukocyte transmigration across the BBB during stroke immune/inflammatory processes could influence inter-endothelial junctional complex function leading to vascular endothelium damage and BBB breakdown. Equally a key component to our mechanism-based thesis is that disruption or dysfunction of the BBB, preceding entry of harmful substances into the brain parenchyma, could be a key initial factor in stroke pathogenesis. Thus, restoration of barrier integrity may have a critical role in preventing stroke progression. Our studies have begun to address these questions, particularly, whether endothelial cell replacement can restore structural and functional properties of the BBB after stroke. Results of this study will provide the basis for pursuing cell therapy both for non-tPA and tPA-treated ischemic stroke patients, as well as for patients with neurodegenerative disorders characterized by BBB dysfunction.

Conclusion

The recognition that t-PA may exacerbate the breakdown of the already vulnerable BBB warrants therapies designed to arrest this BBB dysfunction. Currently, much of the stroke therapy implemented does not consider the capacity of BBB damage after stroke. It is our contention that if EPC transplantation promotes restoration of the vascular endothelium, the clinical effects could be far reaching and substantially help a large population of patients that may be excluded from the current therapeutic window guideline for tPA. Although a plethora of accumulating stem cell research is quickly translating into clinical trials, it is important to gain insights into the mechanisms of action, which will aid in optimizing the safety and efficacy of these stem cells in stroke. There are almost 800,000 stroke cases yearly in the USA but less than 3 percent of these patients benefit from tPA treatment, due to the drug's narrow therapeutic window and its detrimental side effects that can exacerbate stroke injury and counteract the benefits provided by reperfusion of the occluded artery. Accumulating evidence indicates that tPA-induced neurotoxicity may contribute to BBB breakdown and neuronal injury in the acute phase after stroke. BBB damage may result in the formation of severe brain edema over subsequent hours and days in stroke patients. This damage could negatively influence the CNS regenerative processes after stroke. Accordingly, any treatment regimen directed at attenuating stroke deficits should consider the pivotal role of BBB repair in order to maintain CNS homeostasis and enhance neuronal regeneration. In summary, structurally and functionally restoring the BBB in an acute and sub-acute stroke setting may afford therapeutic benefits against stroke. A regenerative mechanism involving the repair of the damaged BBB by EPC is critical to the successful outcome of cell therapy in stroke. Cell therapy tailored at EPC recruitment and/or directed secretion of EPC-soluble factors into the stroke brain stands as a potent strategy for BBB repair in stroke.

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