Stem Cells and Cloning: Advances and Applications

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REVIEW

Brain repair: cell therapy in stroke

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Abstract: Stroke affects one in every six people worldwide, and is the leading cause of adult disability. Some spontaneous recovery is usual but of limited extent, and the mechanisms of late recovery are not completely understood. Endogenous neurogenesis in humans is thought to contribute to repair, but its extent is unknown. Exogenous cell therapy is promising as a means of augmenting brain repair, with evidence in animal stroke models of cell migration, survival, and differentiation, enhanced endogenous angiogenesis and neurogenesis, immunomodulation, and the secretion of trophic factors by stem cells from a variety of sources, but the potential mechanisms of action are incompletely understood. In the animal models of stroke, both mesenchymal stem cells (MSCs) and neural stem cells (NSCs) improve functional recovery, and MSCs reduce the infarct volume when administered acutely, but the heterogeneity in the choice of assessment scales, publication bias, and the possible confounding effects of immunosuppressants make the comparison of effects across cell types difficult. The use of adult-derived cells avoids the ethical issues around embryonic cells but may have more restricted differentiation potential. The use of autologous cells avoids rejection risk, but the sources are restricted, and culture expansion may be necessary, delaying treatment. Allogeneic cells offer controlled cell numbers and immediate availability, which may have advantages for acute treatment. Early clinical trials of both NSCs and MSCs are ongoing, and clinical safety data are emerging from limited numbers of selected patients. Ongoing research to identify prognostic imaging markers may help to improve patient selection, and the novel imaging techniques may identify biomarkers of recovery and the mechanism of action for cell therapies.

Keywords: stroke, cerebrovascular disease, cell therapy, neurological disease

Introduction

Stroke is the most common cause of adult-acquired disability in the developed[1] and developing world.[2] With an aging population, the incidence and prevalence of stroke are predicted to rise.[3] Stroke is an acute-onset clinical syndrome that develops following a vascular insult to the brain. Brain ischemia resulting from thromboembolism or less frequently, in situ thrombosis, constitutes 80%–85%, and hemorrhage resulting from hypertension or vessel wall pathology constitutes 15%–20% of all strokes. Following vascular occlusion, a complex chain of events occurs at a molecular level, leading to irreversible tissue injury, including failure of energy synthesis, loss of transmembrane ionic gradients dependent on active transport, cell depolarization, and excitotoxicity due to the excess release of excitatory neurotransmitters. In the region with severely reduced blood flow (the ischemic

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core), these processes result in rapid cell necrosis affecting all the cellular elements (neurons, glia, and blood vessels). A region around the core (the ischemic penumbra) transiently maintains a collateral blood supply sufficient for cell viability. Restoring perfusion can salvage penumbral tissue, and timely recanalization is the most robust predictor of good clinical prognosis following ischemic stroke.[4] Early thrombolysis with intravenous recombinant tissue plasminogen activator increases the likelihood of recanalization and a recovery to independence defined on scales of disability and handicap.[5] Alternative reperfusion strategies have not yet shown benefit. Secondary processes following ischemic injury and cell necrosis include an inflammatory response, with the activation of microglia, infiltration of tissue by neutrophils and macrophages from the blood, and blood–brain barrier breakdown. Inflammatory mediators can act as chemoattractants for both the endogenous and exogenous cells involved in tissue repair. At the network level, regions of the brain that were previously connected to the infarcted area reorganize, at least in terms of the brain activation patterns seen on functional magnetic resonance imaging (fMRI). Rehabilitation exploits the combination of functional reorganization and adaptation after stroke.[6]

Immediately after stroke, several events, including edema, deafferentation, and inflammation, occur around the infarct, and some early functional recovery can be attributed to the resolution of edema and inflammation. However, this is usually limited, and other processes, including immunomodulation, angiogenesis, endogenous neurogenesis, and altered gene expression, may be involved in the longer-term recovery of function. The apparent translational failure of neuroprotective strategies[7] that aim to interrupt or slow the injurious postischemic biochemical/molecular events may be attributed to various factors, including the heterogeneity of clinical stroke populations,[8] inadequate sample sizes, and dose-limiting drug toxicities. However, recent critiques of the preclinical literature have suggested that the discrepancies between the preclinical and clinical studies are likely also to have arisen from publication bias,[9] the limited replication of results, and experimental methodological flaws that inflated the estimates of effect size and led to the potential selection of inappropriate therapeutic candidates.[10] A series of Stroke Therapy Academic Industry Roundtable (STAIR)[11] meetings produced recommendations on the minimum standards for preclinical evidence that should, ideally, underpin the selection of drug candidates for clinical testing, as well as the clinical trial methods. An equivalent process, entitled Stem Cells as an Emerging Paradigm in Stroke (STEPS), has provided a forum for methodological discussions in the cell therapy field.[12,13]

Overview of stem cell therapy in stroke

Stem cells are undifferentiated cells that have the capacity to self-renew and differentiate into a range of tissues. Stroke therapy has distinct requirements compared with other neurological diseases, like Parkinson's disease or multiple sclerosis, since stroke is nonprogressive, involves a focal loss of tissue of all cell types, and is typically associated with a degree of endogenous recovery. Stem cell therapy is not, therefore, restricted to a paradigm of the replacement of a tissue, or a specific neuronal cell type (the focus in Parkinson's disease, for example), but potentially extends to effects on inflammation, immunomodulation, and the stimulation of endogenous recovery. Cell therapies probably act on multiple mechanisms in ischemic stroke, depending upon the timing and mode of administration; however, unlike neuroprotectant drugs, cell therapies have the advantage that they may be able to respond dynamically to an environment that varies both temporally and spatially after ischemia, rather than targeting a single pathway or mechanism of action. Interaction with the host environment appears to dictate the phenotypic properties of stem cell grafts. Stem cells come from various sources, and although they share some common properties, they also differ in many respects and behave differently in terms of their rate of differentiation, trophic factor secretion, and in their stimulation of endogenous processes when in a pathologic environment. No studies have compared the different cell types in the same experiment.

Endogenous stem cells

Until the middle of the 20th century, it was generally believed that neurogenesis in the mammalian nervous system was restricted to fetal development and that regeneration did not occur in the adult brain. In 1965, Altman and Das[14] first reported postnatal neurogenesis in the rat brain, and by the late 20th century, there was evidence of similar endogenous neurogenesis in humans.[15] In animals and humans, neuroblasts are known to be produced in the subventricular zone,[16] subgranular zone of the hippocampal dentate gyrus,[17] and, albeit controversially, in the newly discovered subcallosal zone that lies between the hippocampus and corpus callosum in rats.[18] Increased neuroblast production following ischemic stroke has been observed in the rat subventricular zone, and cortical neuroblasts have been reported in both a rat stroke model[19] and in human brain biopsy specimens of penumbral

tissue that were acquired for diagnostic purposes after stroke.[20,21] Neuroblast production has also been stimulated experimentally by extrinsic growth factors, like hepatocyte growth factor,[22] and specific molecules, such as statins[23] and fluoxetine,[24] but few of these neuroblasts appear able to migrate to the boundary of ischemic damage,[25] calling into question their functional relevance – amplifying and sustaining this endogenous poststroke neurogenesis response and overcoming the low rate of cell survival may be relevant for functional gains. An improved understanding of the role of changes in the expression of the developmental genes and associated proteins that are observed along the ischemic border after stroke[26] may also be important in developing cell or pharmacologic augmentation therapies that will capitalize on endogenous neuroregenerative capacity.

Olfactory ensheathing cells are a self-renewing population of cells that display the properties of both glia and Schwann cells and are found at the junction between the central and peripheral nervous systems. Their main properties have led them to be studied more in the context of spinal cord and nerve root injuries, but their neuroplastic effects have been tested in murine models of stroke and they have been found to promote neurite outgrowth.[27] Few preclinical studies exist, and their clinical application remains unclear in stroke.[28]

Exogenous stem cells

The application of exogenous cell therapy in neurology began with neurodegenerative diseases, for which fetal ventral mesencephalic tissue was transplanted with the intention of replacement of a specific cell type, such as the dopaminergic neurons of the basal ganglia.[29] Cell replacement for stroke requires the regeneration of multiple functionally specialized cell types, with differing ratios in different brain regions, but extends also to glial cells and blood vessels since the injury involves the entire neurovascular unit.

Neural stem cells

Whether neural stem cells (NSCs) should be defined by their tissue of origin or their capacity to generate neural tissue is not universally agreed.[15] The following discussion considers the tissue of origin to define NSCs. Cells sourced from ectodermal tissue, such as the central nervous system (CNS), have restricted differentiation potential and can further be categorized into embryonic,[30] fetal,[31] or adult,[30] by origin. The use of adult-derived cells does not share the ethical and practical concerns of the use of embryonic or fetal cells. Cells from adult murine brain have been harvested, expanded in culture, and reimplanted as an allogeneic source. Isolated cells can be induced to form neurospheres, which are then expanded in vitro before delivery via various routes, including stereotactic (ST) injection to the brain, and intravenous (IV), intra-arterial (IA) and intracerebroventricular (ICV) administration. The differentiation spectrum of NSCs is restricted to neurons, astrocytes, or oligodendrocytes and can be influenced by intrinsic factors,[32] such as neuron-restrictive silencing factor, and extrinsic factors, such as experimental hypoxia[33] and epidermal growth factors. The transmission of infectious agents by culture media is a concern that can be addressed only incompletely by applying strict Good Manufacturing Practice standards. Human fetal brain cortex cells have been immortalized by the insertion of *c-Myc*[31] and *v-Myc*[34] transcription factor genes, in order to enhance cell survival or allow the regulation of cell replication (for example, where *c-Myc* expression is under the regulatory control of a modified estrogen receptor).[35] The majority of NSC experimental stroke studies have used ST[31,36–38] intracerebral delivery, with implantation ranging from hours[39] to 6 weeks[38] after stroke. Cell migration to ischemic regions has been reported following implantation by ST,[38] IV,[40] or IA[41] routes. ST-implanted human NSCs have migrated up to 1.2 mm in the lesioned hemispheres compared with 0.2 mm in naïve rat brain.[42] Whether more distant migration occurs is unclear. Cell survival varies and depends on the timing and mode of delivery. Following ST implantation, proximity to the lesion influences survival,[38] while very few cells reach the brain following IV administration as they are filtered by the pulmonary vascular bed and sequestered in the spleen.[43] Slightly greater cell survival in the CNS is seen after IA delivery.[44] Although many cells die early after administration, bioluminescent human NSCs ST-implanted 7 days after middle cerebral arterial occlusion (MCAo) have been observed to survive beyond 2 months, with over 50% cell survival confirmed on histology.[45] Surviving cells exhibit a wide spectrum of fates, ranging from 78% remaining in an immature state[36] at week 5, to unquantified numbers of differentiated neurons forming synapses with host cells.[45] The expression of neuronal cell surface markers does not necessarily indicate functioning neuronal tissue, still less, useful integration, and the contribution of the surviving cells to an observed functional improvement is still unclear. A change in neurological or behavioral function has been the preferred outcome, rather than infarct volume, as NSC studies have mostly chosen to implant at subacute time points, when infarcts are well-established. A modified neurological severity score (NSS), which provides a composite score

based on motor, sensory, reflex, and balance responses, has been used commonly in preclinical rodent studies to assess change, reporting significant improvements compared with sham controls, following NSC therapy.[46,47] However, a wide range of behavioral tests has been employed. The reporting of results differs across laboratories,[48] and the reproducibility of tests across observers and also across time has seldom been reported. Despite the lack of clarity regarding the mechanisms of action, NSCs are believed to alter white matter tissue structure, and a noninvasive method to measure this would be valuable. The effects of NSC treatment on white matter reorganization can be monitored by measuring water diffusion[49] using magnetic resonance imaging (MRI) with diffusion tensor imaging (DTI) sequences. Although DTI in small animals is compromised by the relatively lesser volume of white matter present in rodents compared with humans, there are also some advantages to use of DTI, including the ability to apply longer scan acquisition times and higher magnetic field strength. The white matter reorganization observed on histology was coincident with improved fractional anisotropy, and fiber tracking maps revealed similar orientation patterns to that seen on immunohistology.[41]

Mesenchymal stem cells

Since the first bone marrow-derived mesenchymal stem cells (MSCs),[50] many other cell types with similar properties from various tissues, including bone marrow mononuclear cells, adipose-derived stem cells, umbilical cord blood cells (UCBCs), endothelial progenitor cells (EPCs), peripheral blood progenitor cells, cluster of differentiation (CD)34+ cells from placenta, periosteal stem cells, and amniotic fluid cells have all been proposed as potential alternatives. The relative ease of cell acquisition without ethical difficulties has fuelled interest in MSCs, but the specific characterization of MSCs has not been consistent over time,[51,52] making study comparability difficult. In vitro cultures contain a mix of committed and noncommitted progenitors that can form, not only mesodermal, but under certain circumstances, also ectodermal cell types, like neurons, but it is unclear whether the MSCs differentiated along neuronal lines in culture will have the same properties as do NSCs. Human neuronal MSCs, which have the ability to differentiate into neuronal cells following transfection of the Notch intracellular domain, were ST-implanted 4 days after MCAo in gerbils and compared with human MSC. In the human neuronal MSC group, better cell survival and functional recovery were observed despite the absence of synaptic connection between the transplanted and recipient cerebral cells on fluorescence-in-situ-hybridization (FISH), suggesting that the neuronal differentiation did not contribute to the MSC beneficial effects.[53] In experiments with MSCs derived from donor rats,[54] mice,[55] rabbit,[56] (autologous or allogeneic), or humans[57] (xenogeneic), cells have been transplanted by IV,[57] IA,[58] ST,[59] or intracisternal[58] routes into animals, from hours[57] to 1 month[60] after induction of stroke with either temporary or permanent MCAo. Homing of the transplanted MSCs appears to occur via a complex multistep process that includes interactions with the stromal cell-derived factor 1 (SDF-1) (also called C-X-C motif chemokine 12 [CXCL12]) chemokine receptor.[61] Homing signals originate from within the active inflammatory zone in the injured tissue. MSC migration to specific sites has been observed in stroke studies, where they have been found to travel preferentially to the ischemic boundary, following IV[57] and ST delivery.[62] Few cells have been shown to survive in the studies of xenogeneic cell implantation. With no immunosuppression, cell survival of up to 2 weeks has been reported on ST implantation, but the proportion of surviving cells has not been quantified[63] and has qualitatively been described as being a small proportion only. Long-term cell engraftment has not been detected with IV administration on histology.[64] In another study, out of 3×10^6 MSCs delivered IV, only 3% of administered cells expressed neuronal markers in vivo,[65] further supporting the concept that tissue replacement is not likely to be a functionally relevant mechanism of action for this cell type. Trophic factors, such as brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), vascular endothelial growth factor (VEGF), neurotrophin-3 (NT3), fibroblast growth factor (FGF), and thrombospondins, secreted by the MSCs[66,67] in response to the local microenvironment may, along with their stimulation of neurogenesis,[66] angiogenesis,[68] and immunomodulation,[69] underlie functional recovery. Astrocytes are known to maintain normal neuronal function,[70] forming an important pathway for endogenous repair.[71] Exogenous MSCs have been observed to influence astrocyte survival and astrocyte trophic factor gene expression after anaerobic insult, by upregulating several kinase pathways and protein functions.[72] After ischemia, astrocytes form gliotic scar tissue, which may be helpful in limiting tissue inflammation but can impede axonal regeneration. IA-implanted MSCs have shown histological evidence of improved axon-myelin remodeling after stroke,[73] but it is unknown whether this mechanism is relevant in other routes of MSC administration. MSCs naturally adopt different trophic factor expression dependent on the injured host neural tissue.[74] Higher levels of BDNF, NT3, and VEGF

have been detected at the ischemic boundary 14 days after ST human MSC transplantation in rat brains compared with controls that received saline.[75] The expression of VEGF and FGF has been consistently high at the ischemic boundary, potentially driving endothelial cell proliferation and angiogenesis, and facilitating regional blood flow.[76] In contrast with the NSC studies, infarct volume has been the preferred outcome measure for experimental MSC therapy, which has been predominantly administered in an acute or early subacute IV delivery paradigm, with significant reduction in infarct volumes and good correlation noted between histology and imaging measures.[77] Significant improvements have also been reported in behavioral measures, which have included assessments of sensorimotor function, motor coordination, and placing deficits during locomotion (treadmill test),[77] forelimb function and placing deficits (limb placement test),[78] motor coordination and balance (rotarod test),[75] and a composite of motor, sensory, reflex, and balance responses (NSS).[79] Other cell types including UCBCs, EPCs, adipose-derived stem cells, and hematopoietic progenitor cells (CD34+ cells), share some of the properties of bone marrow-derived MSCs and have been found to have similar effects in animal models. In animal experiments, UCBCs respond to ischemic region homing signals, migrate to the lesioned hemisphere following IV administration, and differentiate, as evidenced by immunohistochemical neuronal and astrocytic markers.[80] Some MCAo rat studies have failed to detect IV-administered UCBC in the lesions despite improvement in spontaneous activity and behavioral motor tests, suggesting a trophic factor-mediated response.[81,82] CD34+ cells form a significant component of UCBCs that have been enriched either from the umbilical cord, peripheral blood, or bone marrow, and administered separately. IV administration of CD34+ in MCAo models has shown ischemic border zone neovascularization that has in turn, stimulated endogenous neurogenesis.[83] EPCs represent cells with varying cell expression markers,[84] typically CD34+, CD133+, and kinase insert domain receptor (KDR+) (also known as vascular endothelial growth factor receptor-2), with an angiogenic mechanism of action and found to reduce infarct volumes in rat stroke models when administered IV a day after MCAo.[85] Several Phase I and II MSC clinical trials are ongoing (Table 1).

Embryonic stem cells

Embryonic stem cells (ESCs) are derived from the first stages of embryonic development: the first human ESC lines were established in 1998 from the inner cell wall of the blastocyst stage.[86] Religious and moral objections have been raised to the medical use of embryonic material; however, it is not widely recognized that the ESCs used for medical research are generally obtained from in vitro fertilization programs. Media reports often fail to distinguish ESCs from other stem cell types, leading to public confusion. ESCs are pluripotent and able to differentiate into tissues of all three germ layers. Although at first glance this might appear advantageous, regulatory control over ESC differentiation may be necessary before therapeutic use, since ESCs tend to form teratomas when grafted,[87] with the postischemic environment possibly promoting teratoma formation.[88] ESC studies in animal stroke models have been concerned with mechanistic aspects rather than functional efficacy, and report only isolation, neutralization,[89] and the electrophysiological activity of differentiated neuronal cells.[90] Undifferentiated ESCs grafted into rat brains have differentiated and integrated with host tissues in stroke models,[91] showing improved functional outcomes on the cylinder test, which measures the spontaneous use of forelimbs.[92]

ESCs remain widely researched as a source for in vitro generation of neuronal cell lines for drug screening, mechanistic investigation, or therapeutic use. ESCs can be stimulated to differentiate into specific neuronal populations or glia, with appropriately timed use of growth and inhibitory factors in relevant media and culture conditions. ESCs have been preferentially differentiated to a glutamatergic neuronal phenotype of the auditory nerves, with a view to specific tissue regeneration of the auditory nerve.[93] Similar preferentially differentiated cell cultures can be used for in vitro studies, to investigate several critical stroke-related molecular processes. Such studies provide tight experimental control despite limitations of their ability to investigate the role of cell interactions. The cellular effects of oxygen-glucose deprivation, hypothermia, oxidative stress, and excitotoxicity have been modeled with chosen degrees of injury, helping to improve our understanding of certain key pathological processes.[94]

Induced pluripotent stem cells

Nobel Laureate Shinya Yamanaka and his colleague Kazutoshi Takahashi first demonstrated that differentiated murine cells could be reprogrammed to an embryonic-like state, with cells having the morphology, growth properties, and cell surface markers of ESCs, calling them induced pluripotent stem cells (iPSC). Similar iPSCs were later derived from adult human somatic cells.[95] The Yamanaka method involved the transfection of cells with four key nuclear transcription factors, under ESC culture conditions;[96] subsequent studies have

Table 1 Clinical trials listed on http://www.clinicaltrials.gov (as of August 5, 2013)

Location (trial acronym)	Subjects	Controls	Cell type	Study Phase	Time from stroke onset	Delivery mode	Dose ($\times 10^5$)	Primary outcome	Secondary outcome	ID	Completion date
Neural stem cells (NSCs)											
Glasgow, UK (PISCES)[106]	12	No	Allogeneic NSC: CTX0E03	I	6 mo to 5 y	ST	200	AE	mRS, NIHSS, BI, EQ-5D™	NCT01151124	Mar 2014
Mesenchymal stem cells (MSCs)											
Rio de Janeiro, Brazil[131]	12	No	Autologous BMSC	I	<90 d	IV, IA	5,000	AE	Imaging	NCT00473057	May 2011
Manipal Acunova, India[132]	120	Yes: no intervention	Autologous BMSC	II	7 to 30 d	IV	5,000	BI	NIHSS, mRS	NCT01501773	Oct 2011
San Diego, CA, USA[107]	35	No	Allogeneic BMSC	I/II	>6 mo	IV	1,050	AE	NIHSS, MMSE, BI, GDS	NCT01297413	May 2013
Pune, India (BMACS)[133]	50	No	Autologous BMSC	I, II	NA	Intrathecal (3 divided doses)	1,000	Improvement of body and facial muscles	Improvement in speech, walking, or vision	NCT01832428	Mar 2014
Malaysia[134]	50	Yes: no intervention	Autologous BMSC	II	1 wk to 2 mo	IV	NA	mRS, NIHSS, BI	SIS, QOL	NCT01461720	Mar 2014
Aldagen, USA[148]	100	Yes: placebo	Autologous BMSC (ALD-401)	II	13 to 19 d	IA (carotid)	NA	AE	mRS, NIHSS, BI, EQ-5D™	NCT01273337	Dec 2014
Irvine, CA, USA[135]	40	Yes: placebo	BMSC	I, II	<3 d	IV	NA	AE	Efficacy	NCT01849887	Jan 2015
People's Republic of China[136]	30	Yes: no intervention	Autologous BMSC	I	NA	Intracerebral	40	NIHSS	MRI infarct size	NCT01714167	Dec 2015
Samsung Medical Centre, Korea[137] (STARTING-2)	60	Yes: no intervention	MSC	III	<90 d	IV	NA	mRS	Clinical: mRS, NIHSS, BI, EQ-5D™ Blood: CSF – SDF, S100b, HIF, CD105-CXCR4, BDNF, VEGF. Imaging: rs-fMRI, DTI	NCT01716481	Feb 2016
Grenoble, France[138] (ISIS)	30	Yes: no intervention	Autologous MSC	II	<6 wks	IV	NA	AE	Efficacy	NCT00875654	Aug 2016
MSC alternatives											
Oviedo, Spain[139]	20	Yes: placebo (n=10)	Autologous CD34+	I/II	5 to 9 d	IA (MCA)	1,600	AE	mRS, NIHSS, BI	NCT00761982	Nov 2011
London, UK[140]	10	No	Autologous CD34+	I/II	<7 d	IA (MCA)	NA	AE	mRS, NIHSS	NCT00535197	Jul 2012
Celgene, USA[141]	44	Yes: placebo	PDA001 (human placenta-derived cells)	IIA	1 d and 8 d	IV	2,000	AE	mRS, NIHSS, BI	NCT01310114	Mar 2013
Ageless Regenerative Institute, Mexico[142]	10	No	Autologous adipose-derived cells	I/II	NA	IV	NA	Safety, NIHSS	mRS, NIHSS, BI	NCT01453829	Jun 2013
Madrid, Spain[143] (AMASCIS-01)	20	Yes: placebo (n=10)	Allogeneic MSC-adipose tissue	II	<2 wks	IV	700	Safety/efficacy	NA	NCT01678534	Nov 2013

Houston, TX, USA[144]	30	No	Autologous BM mononuclear cells	I	<3 d	IV	NA	AE	Functional	NCT00859014	Nov 2013
Zhejiang, People's Republic of China[145] (AHSCTIS)	40	Yes: no intervention	Autologous hematopoietic SC	I	NA	IA	40	NIHSS	mRS, NIHSS, BI	NCT01518231	Dec 2013
China Med Uni, Taiwan[146]	6	No	Allogeneic CD34+ UCBC	I	6 mo to 5 yrs	Intracerebral	50	NIHSS	NIHSS, MRI-MRS DTI	NCT01438593	Dec 2013
Athersys – USA and UK[147]	140	Yes: placebo	Multistem™	II	1 to 2 d	IV	NA	AE	mRS	NCT01436487	Nov 2014
Multicenter, USA[149]	18	No	SB623-modified stromal cells	I/IIA	6 mo to 5 yrs	Intracerebral	100	AE	mRS, NIHSS, ESS, FM	NCT01287936	May 2015
Guangzhou, People's Republic of China[150] (AMETIS)	90	Yes: placebo	Autologous BMSC and EPC	I/II	7 d	IV	1,750	AE	mRS, BI	NCT01468064	Nov 2015

Abbreviations: AE, adverse events; BDNF, brain-derived neurotrophic factor; BI, Barthel Index; BM, bone marrow; BMSC, bone marrow-derived stem cells; CD, cluster of differentiation; CSF, cerebrospinal fluid; CXCR4, chemokine receptor type 4; DTI, Diffusion Tensor Imaging; EPC, endothelial progenitor cells; EQ-5D™, quality of life outcome scale; ESS, European Stroke Scale; FM, Fugl–Meyer scale; fMRI, functional magnetic resonance imaging; GDS, geriatric depression scale; HIF, hypoxia inducible factor; IA, intra-arterial; IV, intravenous; MCA, middle cerebral artery; MMSE, Mini Mental State Examination; MSC, mesenchymal stem cells; MRI, magnetic resonance imaging; mRS, modified Rankin Scale; MRS, magnetic resonance spectroscopy; NA, not available; NIHSS, National Institute of Health Stroke Scale; NSC, neural stem cell; QOL, quality of life (outcome scale); rs-fMRI, resting state fMRI; SC, stem cell; SDF, stromal cell-derived factor; SIS, Stroke Impact Scale; ST, stereotaxic; UCBC, umbilical cord blood cells; VEGF, vascular endothelial growth factor.

identified alternative methods.[97] While superficially appealing as a means of obtaining ESC-like cells from adult tissue, the limited yield of these methods, the potential risks of clinical use of material obtained from viral transfection, and the multiple potentially oncogenic transcription factor genes, as well as (for stroke) the time required for culture expansion, all present significant clinical hurdles that are currently being investigated.[98] iPSCs can potentially generate autologous patient-specific cells, avoiding the ethical, moral, and legal issues of ESCs but may share the tumorigenicity issues of ESCs.[87] The intracerebral implantation of undifferentiated iPSCs in a rat MCAo model showed cell expansion to form large tridermal teratomas, with little behavioral improvement compared with controls, despite differentiated neuroblasts and mature neurons being seen in the ischemic lesion.[99] As is the case for ESCs, partial in vitro differentiation may be necessary before therapeutic uses can be contemplated. A recent study that used human iPSC-derived long-term expandable neuroepithelial-like stem cells in a T cell deficient rat MCAo model with a 4-month observation period found no new tumors or transplant overgrowth, suggesting that predifferentiation of iPSCs and the generation of long-term self-renewing neural cell lines may offer an effective strategy for minimizing the risk for tumor formation.[100] The reports of improvement in function, reduced infarct volume, and differentiated neuronal cells with electrophysiological properties and host synaptic connections following the intracerebral implantation of iPSCs derived from human fibroblasts[100,101] are promising, but other studies using ST delivery of iPSCs have reported no functional improvement.[102]

Stem cells and the immune system

Transplanted stem cell survival may be influenced by host immune responses, but the transplanted cells may themselves modulate the host inflammatory microenvironment after stroke. The immunogenicity of allogeneic stem cells varies according to the expression of their major histocompatibility complex (MHC) I and II and other molecules that stimulate host CD8+ or CD4+ T cells.[103] MSCs express very few MHC antigens, but cell surface marker expression may be modified by the host environment, and the lack of in vitro immunogenicity may not therefore be informative about the potential for problems in clinical use. However, to date, there have been no reports of cell-related adverse events or tumorigenesis following autologous MSC administration in the small number of early Phase I clinical trials in stroke[104] and multiple sclerosis.[105] Two clinical trials, of allogeneic NSC[106] (NCT01151124) and MSC[107] (NCT01297413) lines for the

treatment of stroke with no coadministered immunosuppression, are currently investigating safety outcomes, including clinical, laboratory, and imaging markers. Although there is evidence that adult stem cells have an inherent immunologically privileged status and are capable of escaping rejection,[108] it is unclear whether their MHC expression is altered by exposure to proinflammatory cytokines, such as occurs in ischemic tissue injury. ST-implanted neural progenitor cells have been observed to have low immunogenicity as they are not exposed to systemic immune surveillance, but the blood–brain barrier is damaged after stroke and the CNS probably does not retain this status. There are suggestions that low immunogenicity could be a unique property of NSCs, based on a lack in upregulation of the immunological response to transplantation of murine NSCs, and the lack of difference observed in animals, whether or not immunosuppressed, 2 weeks postimplantation and 4 weeks post-MCAo.[109] Some xenogeneic animal stroke studies have coadministered immunosuppressant drugs on the assumption that the recipient species would reject donor cells of human origin. Whether or not xenogeneic studies necessitate immunosuppression is still unclear. Many studies have not reported the use of immunosuppression or have not considered studying its effects in detail.[36,40,45] Immunosuppressant drugs have independent neuroprotective effects in animal models of stroke, and their use was identified as a significant factor in modifying effect size estimates in a meta-analysis of animal studies.[110]

Transplanted stem cells initiate a dynamic sequence of host immunomodulatory actions on exposure to the host inflammatory microenvironment. They not only integrate and differentiate but also home in, extravasate into the CNS, and modulate immune responses in situ.[111] NSCs are reported to show more tropism towards inflammatory sites than do MSCs.[112] Both NSCs and MSCs exhibit host immune modulation in vivo. MSCs release neurotrophic factors, such as BDNF, provide trophic support for vulnerable neurons in the ischemic penumbra, support endogenous oligodendrogenesis, and regulate anti-inflammatory responses, leading to enhanced tissue sparing.[65] NSCs attenuate brain inflammation, modulate microglia activation, limit demyelination, and promote host-driven repair.[113]

Clinical trial design

Ideally, preclinical evidence of efficacy, information on the optimal timing and mode of delivery, and toxicity (including tumorigenesis and possibly gene silencing studies) should be considered in clinical trial planning. The STEPS[12,13] meetings have suggested essential minimum criteria for the design of cell therapy stroke trials, by incorporating general principles from the earlier STAIR proposals that primarily concerned pharmaceutical development.[11] Although these recommend that preclinical studies include more than one strain of rodent, animals of varying ages, and that there be independent confirmation from one or more laboratories, in reality these recommendations are rarely followed due to high costs and potential commercial restrictions, and preclinical information may thus be limited.

The selection of an appropriate target stroke population will be influenced by the phase of study, expected mode of action of the cell therapy under study, and preclinical data. For studies primarily collecting safety data, chronic stroke patients with a broad range of severity who are not within the natural recovery period are likely to be candidates. The dose of stem cells for humans would usually be estimated based on animal studies and will need further human testing to define the maximum tolerated dose, minimum effective dose, and ideally, a dose-response curve. For a safety trial, an ascending dose design could be incorporated, especially for ST-delivered cells. For studies gathering efficacy data, subjects are likely to be in the acute or subacute stage after stroke, having deficits that are measurable by well-validated clinical scales, and whose natural evolution and variability over time after stroke are understood. Biomarkers, such as imaging, may offer greater biological confidence in the effects of treatment, with sample sizes that are smaller than are necessary to distinguish differences in the clinical disability scales; imaging markers should correlate with clinically relevant measures. For long-term safety follow up, the prevalence of significant comorbidities in stroke populations and the intensity of observation in a typically disabled and elderly population need to be considered in order to minimize trial subject attrition.

Stroke lesion sizes and locations are heterogeneous, and there is considerable interindividual variation in the neuroanatomical systems involved. Experimental stroke induction is a more controlled event, intended to produce a consistent lesion size and distribution. Anatomical characterization will thus play a significant role in patient selection in trials, not only from the perspective of surgical planning and feasibility for studies using delivery by ST implantation, but also, more generally as a prognostic marker. For example, corticospinal tract integrity predicts motor impairment[114] and the probability of motor recovery.[115] Likewise, the timing of the ST intervention can be challenging in the acute stage, when lesion size varies considerably with improving edema and anatomical remodeling. While IV delivery is more straightforward from this aspect, a persistent occlusion of the target artery compromises IA cell delivery and may

significantly reduce cell penetration or compromise survival at the target site. Timing will also be influenced by knowledge of the natural course of recovery and how this aligns with the chosen cell's mechanism of action to maximize effect.

The feasibility of blinding patients and trialists to treatment allocation varies by the treatment delivery route and the requirement for placebo controls. Both placebo and blinding are relatively easier for IV therapy than for more invasive delivery routes since there are procedural complications from either IA or direct intracerebral delivery. Whether the scientifically rigorous inclusion of placebo controls to permit double blinding is sufficiently justified instead of potentially less hazardous sham alternatives that could yield a single-blind study (for example, a small incision in the groin rather than an IA placebo injection, or a scalp incision or burr hole rather than an injection of placebo fluid into the brain) may depend on the stage of research and the procedural risks. Functional change, for example, serial clinical scores or functional brain imaging, including both pre- and post-treatment periods, may reduce variance. Domain-specific endpoints, as suggested by Cramer et al,[116] may be more relevant than broad global outcome scales that traverse multiple neural systems; at the same time, they may restrict the trial entry criteria to those patients with very specific deficits, and thus reduce the generalizability of trial results (for example, motor outcome endpoints necessitate motor deficits at entry but a positive effect may arguably not be applicable to speech deficits).[117] Imaging-based outcomes may help to compare metrics, as they could be applied to both humans and animals. The use of imaging biomarkers for recovery prediction is promising, and these are currently being tested. The major confound of providing routine physical rehabilitation treatment in stroke recovery trials is an unresolved issue. While some studies of therapy inputs for specific clinical problems have identified dose-response relationships,[118,119] for many routinely applied interventions, the efficacy or dose relationship is unknown. Even where evidence supports the therapy interventions, few clinical services deliver the optimal dose routinely, and there is enormous variability across sites and healthcare systems. Since animal studies have reported that concomitant specific physical rehabilitation may be a prerequisite of stem cell efficacy, this represents a major challenge in clinical study design.

Advances and future prospects in stem cell therapy for stroke

Many clinical investigations are documented on trials databases as planned or underway (Table 1), but the great majority of these studies are safety and tolerability studies, with small sample sizes and unspecified control groups. A wide range of cell types is being investigated, but most studies plan autologous bone marrow-derived cell administration by intravascular routes at subacute time points. These studies, if completed, will contribute valuable safety data that is a necessary prelude to large-scale efficacy trials, but ultimately, large randomized controlled trials with broad clinical endpoints will be required to judge the balance of risks and benefits.

The genetic modification of stem cells (for example to enhance the delivery of trophic factors, like BDNF[120] or VEGF,[121] or to address large scale manufacturing through conditional cell immortalization[30]) may offer advantages for allogeneic cell therapies. The allogeneic approaches offer the hypothetical advantage of immediate "off the shelf" availability, which is not possible with autologous cells, even if cells are not culture-expanded prior to administration. Laboratory research into the use of nonviral vectors for stable modification of cells, in vivo cell tracking, and the modification of stem cell gene expression profiles, is ongoing and will improve our understanding of cell function.[122] Tissue replacement as a therapeutic goal is almost certainly beyond the scope of the current therapeutic approaches in stroke, but the development of extracellular matrix bioscaffolds, to provide structural support for human NSCs, is a promising and potentially relevant approach for chronic stroke and other forms of brain injury.[123] The concept of stem cell–secreted extracellular membrane vesicles, providing extracellular waves of information capable of inducing multiple functional responses in adjacent and distant target cells, has emerged recently; the relevance of the bidirectional genetic information exchange between stem and target cells via MSC-secreted extracellular membrane vesicles[124] is under investigation as a possible means of modifying graft–host interactions.

Parallel advances in biomaterial engineering and nanotechnology could provide an inert scaffold for ex-vivo stem cell expansion and intracranial delivery,[125] and may in future address the limitation, for current cell therapy paradigms, of the major loss of brain tissue after stroke that leaves only a cystic cavity.

Applying novel imaging techniques to monitor stem cell effects and identify biomarkers is likely to be the key to the neurological application of cell therapies. Conventional structural imaging is unlikely to be helpful, but modalities, such as motor task fMRI can predict treatment response[126] and provide a measure of the balance of interhemispheric control,[127] and DTI can provide information on axonal integrity, which correlates with functional recovery.[128] Approaches such as resting state fMRI may allow the

assessment of the effect of stem cells at a network level on either hemisphere.[129] Multimodal approaches[130] combining fMRI and DTI are advancing, and more work with stem cell–treated subjects will improve the use of imaging-based biomarkers for patient selection, baseline stratification, and outcome assessment.

Conclusion

Contrary to long-held beliefs, we now know that the brain is highly malleable after an ischemic insult. Endogenous neurogenesis, angiogenesis, and synaptogenesis occurs in humans, albeit at a rate that is able to provide only partial functional recovery in the majority of cases. Cell therapy offers a potential for multimodal action that is promising within the domain of brain repair therapies. Despite the almost certain publication bias in animal stroke studies, stem cell experiments have shown evidence of cell migration to the lesion, survival, and varying degrees of differentiation. Both tissue-specific NSCs and non-tissue-specific MSCs have been associated with significantly improved behavioral outcomes. A comprehensive understanding of their mechanism of action is lacking, but tissue replacement is now believed likely to constitute only a minor contribution (if any) to the therapeutic effect. Accordingly, a cell type's capacity to differentiate along specific pathways is likely to be a less relevant consideration. The multiple mechanisms of action of stem cells include the secretion of trophic factors, immunomodulation, and anti-inflammatory effects. The great majority of the early cell therapy clinical studies have involved adult-derived cells of either autologous or allogeneic origin, and no major safety issues have been identified to date, although the numbers of subjects have been extremely small and follow-up periods limited. Several clinical trials are ongoing or planned, mostly using MSC cells delivered by IV infusion.

Disclosure

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