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Restorative Therapy in Stroke

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Abstract

A variety of therapeutic approaches that could be considered neurorestorative are currently in clinical trials after stroke. There are essentially two varieties of restorative approaches. One is cell-based and includes stem cell transplantation with and without augmentation with growth factors and other variety is the pharmacological approach. These strategies are being explored for the ultimate aim to regain maximum restoration possible and eventual complete normalcy of function. Functional recovery post stroke may require new synaptic connections within and away from the damaged tissue. In an infracted area, the ischemic core may not respond to any pharmacological or rehabilitative intervention. For these reasons, the prospects of repairing the neuron system, using various putative restorative strategies seems promising and urgently required for further exploration, refinement and optimization. Ongoing animal and human trials have largely helped in burgeoning our hopes on this method of restorative therapy after stroke.

Keywords: Stem cells; Stroke; Neuroregeneration; Brain plasticity; Restorative therapies

Introduction

Increased understanding of pathogenesis, path physiology of stroke in the last few decades has paved way for path breaking advances in restorative medicine with special application to recovery after stroke [1]. These advances has helped launch a new stroke era, relinquishing the nihilism of the past and entering a new momentum of hope and aggression to salvage the critically perfused brain parenchyma as well as harness the latent capability of the brain to recover and regenerate, termed as neural plasticity [2].

The injury, repair and recovery after stroke have been extensively defined. The first epoch is related to acute injury and takes place in the first initial hours after stroke when changes in blood flow, edema, metabolism rate and diaschisis occurs. A second epoch is related to repair, which starts days after stroke and lasts for several weeks and is referred to as endogenous repair suggesting a golden period for initiating restorative therapies. A third epoch occurs weeks to months after stroke when spontaneous recovery gains have plateaued and this represents a stable but modifiable early and late chronic phase [3].

The ultimate aim of any therapeutic strategy is the maximum restoration possible and eventual complete normalcy of function. The non-regenerative capability of the injured adult brain has been challenged in recent years and neural plasticity has been observed experimentally in both global and focal brain ischemia in animal models. Neuroimaging studies in stroke patients indicate altered post stroke patterns suggesting functional reorganization. However, whether neuro - genesis increases in response to brain lesions and whether same stem cells or progenitor cells present in brain be used for transplantation are potential questions that need to be answered. Recent studies have shown *in-vivo* differentiation of progenitor cells into neurons in adult human dentate gyrus. Functional recovery may occur in a small or localized brain injury using rehabilitation measures, but for large ischaemic strokes, the restoration may require new synaptic connections within and away from the damaged tissue. Considering the relatively poor capabilities of neural self-regeneration, this seems quite impossible. In an infarcted area, the ischemic core may not respond to any pharmacological or rehabilitative intervention. For these reasons, the prospects of repairing the neuron system, using cell transplantation seem promising and may offer a unique approach for brain repair and restoration of function [1,4]. Considering the fact that the neuronal circuitry is a complex array of neurons and connections and the prospects of this technique at first thought seem remote, yet, the growing evidence from animal models and small clinical trials has suggested the possibility of reconstruction of neuronal network, making the aspect of restorative medicine significantly promising.

The injured cerebral tissue in various ways reverts to a quasi-ontogenous or developmental state, expressing genes and proteins that are developmental and that lead to brain remodeling. In this quasi-developmental state, angiogenesis, neurogenesis, and synaptogenesis are evident [5]. These restorative processes that are interdependent essentially remodel the brain and lead to improved neurological function. However, these restorative processes are often inadequate to fully restore neurological function and many stroke patients are left with severe neurological deficits. The essential question therefore is, whether we can amplify these restorative processes so that neurological function can be enhanced post-stroke [6,7].

A variety of therapeutic approaches that could be considered neurorestorative are currently in clinical trials after stroke. There are essentially two varieties of restorative approaches. One is cell-based and includes stem cell transplantation with and without augmentation with growth factors and other variety is the pharmacological approach. These include, statins, erythropoietin and analogs, human chorionic gonadotropin, growth factors, and agents that increase cyclic GMP [8,9].

Numerous pharmacological agents which may mimic or reflect developmental processes which promote brain recovery are under investigation [10]. Trophic factors such as brain derived neurotrophic factor (BDNF), hepatocyte growth factor (HGF) and granulocyte macrophage colony stimulating factor (FM-CSF) and other agents such as minocycline have been demonstrated to provide restorative therapeutic benefit in preclinical studies and have moved into clinical trials [11].

Chopp et al. have pioneered the use of phosphodiesterase 5 inhibitors, statins, and agents that increase highdensity lipoproteins and hormones such as thymosin beta 4, erythropoietin and carbamylated erythropoietin for the treatment of stroke and neural injury [12]. They also recently published literature on the use of multifactor restorative agent cerebrolysin for stroke therapy.

Role of Stem Cell Therapy in Post- Stroke Recovery

Stroke poses special conditions that impact the potential success of transplantation to enhance neurological recovery, including the anatomy and time of stroke, the vascular supply, site of implantation, and type of patients enrolled in clinical trials [3]. An infarct might involve the thalamus, hippocampus, and striate visual cortex affecting 3 or more very different neuronal populations. Besides, oligodendrocytes, astrocytes and endothelial cells are also affected. Reconstitution of the complex and widespread neuronal-glial-endothelial interrelationships may require cells for transplant to initially remain immature and phenotypically plastic to differentiate into appropriate neural, glial and endothelial cell types depending on the ectopic site. If white matter is destroyed in a stroke, cell implants may not produce functional connections with axons that can penetrate through the scar tissue of a chronic infarct.

There is uncertainty about the mechanism(s) by which cell transplantation might improve stroke deficits. Transplanted cells would ideally replace cells that are damaged by ischemia and take over function of these cellular elements. However, it is also possible that transplanted cells secrete trophic factors that help to maintain marginally surviving cells or otherwise enhance the local environment sufficiently to improve function. Transplantation might also conceivably produce a host reaction that could include sprouting of new axons and synapse formation [4].

Cell Types and Sources

The therapeutic effects of implanted neurons or neuronal precursors are likely to be successful if they have the capacity to survive, sustain, proliferate, transform into relevant cell types and integrate into host cytoarchitexture and release relevant hormones or neurotransmitters which ultimately would transform into functional benefit. Following cell types have been studied as potential candidates for neural repair in ischaemic stroke.

a) Embryonic/Fetal cells

Fetal tissue has been the major source of cells for transplantation in animal models of stroke. The gestation age of 14-20 days is generally used in animal models. For fetal hippocampal and cortical donor cells, days 18-20 and for fetal striated cells, less than 16 days have been generally used in majority of studies. Since there are major ethical and legal issues governing the use of "human fetal embryonic tissue", other cell sources are being seriously considered and investigated [13,14].

b) Allogenic cells/Porcine cells

Pigs are useful as donors as they are non – endangered species and produce large litters as opposed to non – human primates. Transplantation of fetal cells from primordial striatum of porcine origin, known as lateral ganglionic eminence (LGE) was shown to improve function in rat ischemic models [15-17]. The likelihood of graft rejection in humans is of potential concern and strategies need to be devised to overcome this. The risk of host contamination of viruses is of immense concern. It has been reported that porcine endogenous retrovirus particles (PERV) could be released from the porcine cell lines and can infect human cell lines. Since then a debate on PERV infection from xenotransplantation or its integration into human retrovirus, with resultant novel mutations has been ongoing. Guidelines call for regular monitoring of patients undergoing xenotransplantation.

c) Immortalised cell lines

In view of the ethical difficulties in transplanting embryonic cells and technical problems in xenotransplantation, alternative sources of graft cells have been devised. One of these cell lines, called "immortalized cell lines" have been an important technical advance in the field of neurotransplantation. These cell lines are derived by infecting neuroepithelial precursor cells from predefined CNS regions before their terminal mitosis, with a retrovirus encoding an immortalizing oncogene. Data from studies suggest that the neural precursor cell lines are plastic, and have ability to differentiate into multiple lineages *in vitro* and can respond to local micro environmental cues [13]. The advantage of establishing an immortalized cell line is in providing an unlimited number of identical cells from a single cell propagated in culture, higher level of neurotransmitter production using genetic manipulation, better pooling and sorting of viable cells, screening for infectious diseases and efficient planning of surgical procedure.

d) Spontaneously arising neural cell lines or neuron like cells

Neuroblastomas and glioblastomas are the chief spontaneously arising neural cell lines. These contain cells of mixed population which are often undefined. Embryonal carcinoma (EC) cells are derived from spontaneously occurring testicular germ cell tumors and can differentiate into both neural and non – neural cells. In response to therapy with retinoic acid, the mouse derived EC cell line (P19), differentiates into neurons, astrocytes and oligodendrocytes. However, neural transplantation studies on rat striatum showed that these cells tend to retain their original characteristics established *in vitro* and have phenotypic plasticity *in vivo*. Although tumorigenicity has not been observed, the risk is potential once transplanted.

N–Tera–2 Cells were derived from human testicular germ cell tumor, years ago. Also called LBS-neurons (after Layton Bioscience Inc. Ath. Cal), the credit of development and patenting of the process to cleverly transform this rapidly dividing cell line into fully differentiated non – dividing neurons goes to researchers at University of Pittsburg, Pennsylvania. Upon several weeks treatment with retinoic acid (an agent known to produce maturation of cancer cells into their normal looking non – cancerous equivalents) and mitotic inhibitors, an enriched population of post – mitotic differentiated neurons known as NT2N or HNI cells, showing an exclusive commitment to neural lineage were produced [18]. They have been seen to closely resemble neural precursor cells, and express cell surface markers and

cytoskeletal proteins unique to neural stem cells. They represent a well characterized and unlimited source of human neurons for transplantation that can be reproducibly generated. NT2N cells are "Neuron like Cells" as they have a symmetrical morphology, elaborate an extended axon and elongated dendrite. These cells can express neurotransmitters, functional glutamate receptors, calcium channels and proteins capable of secretory activity and synaptogenesis [19]. The ready constant availability of cryopreserved pure neurons, has made the NT2N cells an attractive graft source and trials in animal studies and initial results in ongoing clinical trials in humans are encouraging.

e) Adult stem cells

The long stranding dogma of adult mammalian brain lacking neurogenesis and evidence of progenitor cells, has been recently challenged by studies showing continuous neurogenesis in olfactory bulb, hippocampus and dentate gyrus, from the neural stem cells (NSCs). These NSCs are defined as undifferentiated cells that are able to self – review as well as generate the three major cell types that constitute the CNS: neurons, astrocytes and oligodendrocytes, signifying their pluripotent nature [20].

In adult animals stem cells are present in organs like bone marrow, skeletal muscle, intestine, liver, peripheral nervous system and retina etc. These features have lead to many studies aimed at characterizing, isolating, expanding and transplanting these fascinating cells. Whether neural stem cells meet all these criteria is still unresolved. It is likely that cell lineages generated from NSCs differ among stages and regions of the CNS, e.g in the cortex there may be selective progenitor cells giving rise to neurons (neural progenitor cell) or astrocytes and oligodendrocytes (glial progenitor cell). There are selective marker molecules for NSCs e.g; mushashil (RNA binding protein), nestin (intermediate filament) and the members of SOX family, but are not cell surface antigens unlike haematopoetic cells [21].

The sub ventricular zone (SVZ) and ependymal layer also corresponds to neurogenesis site in adult brain. Regeneration may also be made effective by stimulating the endogenous neural precursor cells or stem cells by injury, as has been shown in studies, either spontaneously or by using exogenous stimuli like neurotrophic factors (BDNF) administered intraventriculary or by infusion. Thus, it is likely that adult brain parenchyma may recruit and/ or generate new neurons, which could replace the lost neurons.

f) Bone marrow stromal cells

The bone marrow stromal cells (BMSCs) provide structural and functional support for the generation of blood cell lineages from haematopoeitic stem cells e.g; fibroblast reticular cells, adipocytes, macrophages etc, and under specific conditions differentiates into a variety of tissue, e.g bone, cartilage, muscle, glia and neurons. When exposed to epidermal growth factor or neurotrophic factors like BDNF *in vitro*, or cultured with neural cells, human BMSCs differentiate into cells, expressing neural precursor cells (NPC) markers [5]. The advantages with these cell lines seem many. Obtaining marrow cells would be easy and expanding them in culture would not be that difficult. Using patient's own BMSC would theoretically eliminate the risk of rejection. However, differentiation mechanism for these cells is poorly understood. Whether these cells truly produce neuronal synaptic network with plasticity or produce trophic factors alone is questionable and speculative. Issues like long-term survival, safety, plasticity and behavior of BMSCs need further evaluation before clinical use (Figures 1-5).

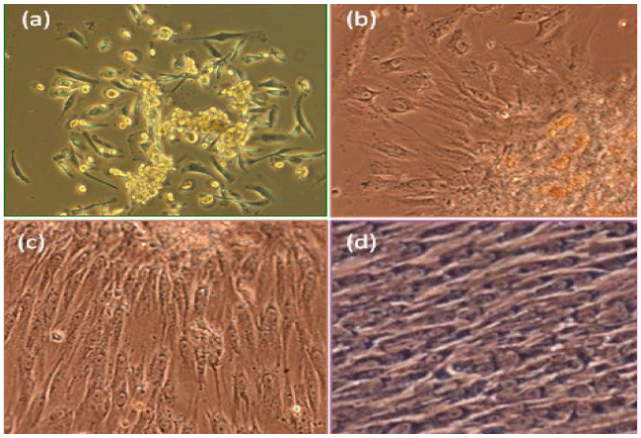


Figure 1: Expansion of mesenchymal stem cells on day 3 (a), 7 (b), 10 (c) and at confluency (d).

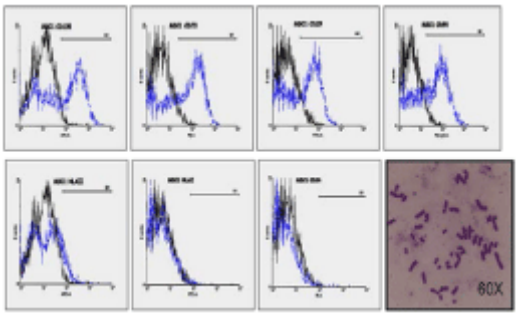


Figure 2: Mesenchymal stem cell markers using flow cytometric showing CD 29, CD 90, CD 105, HLA II phenotypes.

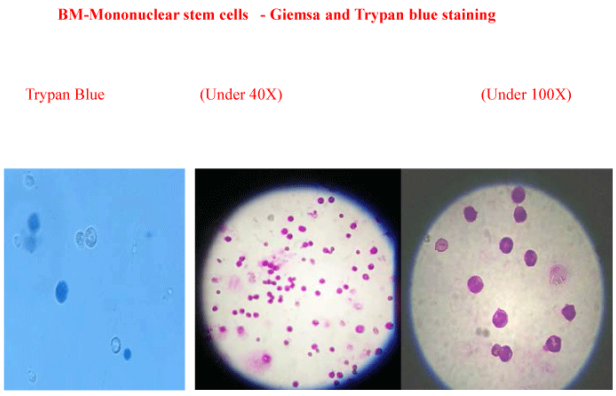
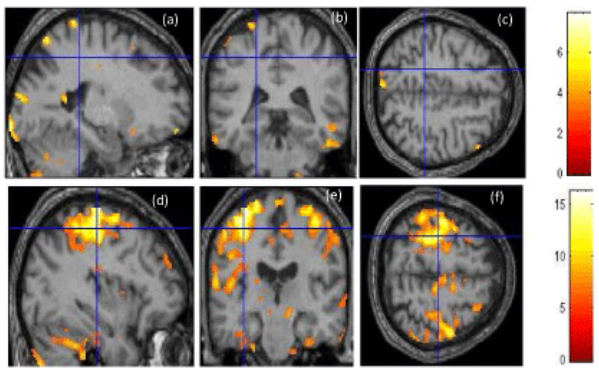
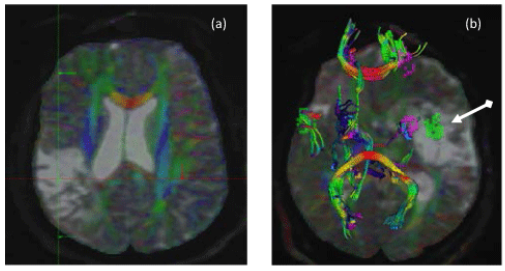


Figure 3: Bone marrow derived mononuclear cells.



Activation maps superimposed on whole brain T1 images for (a-c) Pre treatment activation, (d-f) Post stem cell treatment activation in sagittal (a,d), coronal (b,e) and axial (c,f) orientations.

Figure 4: fMRI images.



(a) Colour coded FA maps of a right temporo-parietal infarct.
(b) The FA values were considerably reduced on the right side as compared to the left.
(b) Tractography of a left MCA infarct.
The corticospinal tract on the left side was disrupted as shown by the arrow.

Figure 5: DTI images.

g) Umbilical cord blood cells

Human umbilical cord blood may also harbor cells (human umbilical cord blood cells [HUCBs]) capable of differentiation into neural lineages. When exposed to nerve growth factor and RA, the derived umbilical cells produce progeny that show positivity of neural and glial cells markers. However, biology of the cells is currently poorly understood, and it is likely that positive effects of these cells are related to their neurotrophic action, rather than actual neuronal circuitry formation [22,23].

Possible Mechanisms of Stem Cells in Restorative Medicine

It is likely that therapeutic effects of the implanted neurons or their precursors, would be dependent upon their functional and structural integration into the brain tissue. It is likely that transplanted cells release neurotransmitters or neurotrophic/ neuroprotective factors which counteract degeneration or promote regeneration. Even transplanted glial cells have been used to modify response to injury and assist in structural repair and promote remyelination [24]. Studies using bone marrow stromal cells or umbilical cord blood cells as potential donors have shown functional improvement in behavioral recovery in animal models within days of transplantation. This raises issues whether recovery observed in such short periods is related to release of trophic factors rather than engraftment and differentiation of transplanted cells into mature neurons and / or glia [25,13]. The functional benefits after neural transplantation are likely to be mediated by one of the following mechanisms.

1. Neurotransmitters released from the graft tissue act on the afferent deprived limb of the post synaptic receptors.

2. Release of the neurotrophic / growth factors (brain derived neurotrophic factor [BDNF], glial derived neurotrophic factor [GDNF], nerve growth factor [NGF] etc) acting as local pumps to support cell function and to prevent cascade of apoptosis. Regenerating neuronal population further prevents subsequent cell death.

3. Reestablishment of local interneuronal connections and synaptic connectivity between the host and graft.

4. Cell differentiation and integration.

5. Improvement of regional oxygen tension.

6. Limit glial reaction and prevent retrograde degeneration. Possibly, the overall success of functional outcome is mediated by a combination of the above mentioned factors.

Role of Vascular Endothelial Growth Factor and Other Growth Factors in Post-Stroke Recovery

Angiogenesis is the key feature of neuronal post stroke reorganization and stroke recovery. Brain ischemia itself induces angiogenesis through hypoxia inducible factor 1 (HIF-1), a transcription factor that responds to the changing intracellular O2 concentration and induces erythropoietin (EPO) expression [26,27]. Angiogenesis is activated through release of polypeptide growth factors and cytokines and specific up-regulation of the angiogenic factors involves transforming growth factor-beta (TGF-β), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF-2) in response to ischemic stroke, but VEGF is the most potent hypoxia inducible angiogenic factor amongst all and is secreted by endothelial cells and pericytes [28]. VEGF is up-regulated by other growth factors within hours of stroke and has a strong influence on growth of new blood vessel in the injured areas of the brain. Its production constitutes adaptive response to hypoxia, which promotes angiogenesis in post stroke events and eventually leads to functional recovery [29].

Role of VEGF in Post-Ischemic Stroke Recovery

Endogenous VEGF

In the ischemic brain, the macrophages, neurons and glial cells appear to contain VEGF. Macrophages in the periphery and in core of early stage of infarct become the first main source of VEGF. Macrophages also participate in a angiogenesis; a macrophages derived peptide PR39, inhibited the ubiquitin-proteosome dependent degradation of HIF-1 alpha protein, resulting in accelerated formation

of vascular structure *in vitro*. These neurons could secrete VEGF under hypoxic conditions along with endothelial cells [30].

Many cytokines and growth factors have been shown to modulate VEGF gene expression. IL-6 produced locally by resident brain cells plays an essential role in post stroke angiogenesis. Increased expression of these genes leads to increased angiogenesis and improved cerebral blood flow during delayed phase of stroke, thus conferring improved long term outcome with reduced lesion size. IL-6 preconditioning of neural stem cells was found to induce secretion of VEGF from these stem cells through activation of signal transducer and activation of transcription. Platelets also contribute to tumor induced angiogenesis as platelets are the carrier of angiogenic growth factors including VEGF [31,23]. Certain indirect angiogenic cytokines such as TGF-β1, may act via induction of bFGFs and VEGF gene expression in the cells resident near endothelial cells *in vivo*. Hypoxia constitutes a potent stimulus for VEGF gene expression but does not regulate bFGF under the same experimental conditions.

EPO plays an important role in angiogenesis through up regulation of VEGF/VEGF receptor system, both directly by enhancing neovascularization and indirectly by recruiting endothelial progenitor cells (EPCs) [32]. It also significantly increases brain derived neurotrophic factor (BDNF) in ischemic area. Endogenous prostaglandin E2 also up regulates VEGF expression by activation of EP4 receptors and heals indomethacin-induced small intestinal lesions.

Exogenous VEGF

Hypoxia itself induces an increase of VEGF expression in ischemic area of brain but this endogenous VEGF secretion is inadequate to entirely protect the brain injury. VEGF plays pivotal role in angiogenesis *in vivo* thus therapeutic cerebral angiogenesis to enhance collateral vessel formation in ischemic area using VEGF which is a specific mitogen for endothelial cells can be a potential method for cerebral revascularization. Itraventricular injection of VEGF antibody increased the infarct volume after focal cerebral ischemia in rats, suggesting that expression of neural VEGF may be one of the neuroprotective mechanisms [30,31].

Topical application of VEGF to the cortical surface as well as intramuscular injection of VEGF reduces infarct volume can brain edema after temporary middle cerebral artery occlusion (MCAO) and this effect is mainly due the neuroprotective function of VEGF in cerebral ischemia. Determination of the optimal dose of VEGF, route of administration, time of administration and its combination with other growth factors will provide more information on the optimal method of using these growth factors for post stroke recovery.

Post - Stroke Release and Action of VEGF in Human Studies

In humans, expression of VEGF was found to be significantly increased after acute ischemic stroke. VEGF reaches its peak 7 days after stroke and remained elevated up to 14 days. Mean VEGF expression was lowest in serum of patients with small infarct, increased in moderate infarct and was greatest in large infarct, which indicated that VEGF could be used as a biomarker for the size of the infarct.

Serum VEGF levels also correlated with the long term prognosis in acute ischemic stroke patients. VEGF levels increased in acute stage were found to be proportional to improved NIHSS scores after 3 months. Thus VEGF levels could be used as biomarkers in long term prognosis of stroke as well [33,34].

Exercise and VEGF

Functional capacities in acute stroke patients have a major impact on the motor function, balance, mobility and activity of daily living [35,36]. Regular exercise after stroke led to functional recovery which sustains for long. Exercise induces neurogenesis and angiogenesis through growth factors cascade [37-39]. Endurance exercise, i.e., running up regulates BDNF and synapsin 1mRNA which helps to facilitate better outcome in patients with stroke. Exercise preconditioning up regulates VEGF which further regulates expression of matrix metalloproteinase (MMP2). MMP2 facilitates conversion of pro-NGF and pro-BDNF into NGF and BDNF respectively. Altogether this pro-angiogenic factor leads to repair and restoration process of brain post ischemic insult. Exercise also strengthens the micro vascular integrity after cerebral ischemia and up regulates endothelial nitric oxide (NO) synthesis, which improves endothelium function by again up regulating VEGF expression [40-42]. Early exercise after MCAO improves blood flow capacity in the ischemic cortex and reduces infarct volume and promote functional recovery. Exercise therefore, modulates endogenous angiogenic mechanisms and exert its role in neurovascular remodeling mainly through VEGF which offers a potential breakthrough for development of new method for long term recovery after stroke [43].

Exogenous VEGF with Stem Cells Transplantation Post Stroke

It is hypothesized that bone marrow derived stem cells may act through secretion of different cytokines and chemokines such as VEGF, Insulin like growth factor - 1 (IGF-1), endothelial growth factor (EGF), angipoietin-1, EPO etc., which are known to enhance wound healing in ischemic area. It is hypothesized that transplantation of the VEGF gene modified stem cells may provide more potent autologous cell transplantation therapy for stroke than transplantation of stem cells alone. In animal models of stroke, telomerized stem cells transfected with BDNF, Glial derived growth factors (GDNF) and ciliary neurotrophic growth factor genes using fiber-mutant adenovirus vectors, leads to significant functional recovery and reduces ischemic damage with more efficacy than treatment with stem cells alone and effect can be seen even when it is applied 6 hours after infarction. This method also maintains exceptionally high level of neurotrophic growth factors, e.g., BDNF during critical post-ischemic period which contributes to enhanced neuroprotection. Thus growth factors and stem cells work synergistically in functional restoration and angiogenesis post-stroke.

Transplanted stem cells in animal stroke models, secreted VEGF which induced neovacularization in spatio-temporal manner in peri-infarct region at 2 weeks post transplantation and influenced tissue already undergoing repair and revascularization and restored Blood Brain Barrier (BBB) on its sub-acute delivery.

Study on Role of Intensive Physiotherapy and rTMS on Growth Factors as Biomarkers for Stroke Recovery

This is an ongoing research by the authors examines the up regulation of growth factors (VEGF) after acute ischemic stroke and its

correlation with clinical recovery as measured by stroke outcome scales. It also examines the effects of recurrent Trans cranial Magnetic Stimulation (rTMS) (1Hz) and correlates the expression of VEGF in the groups receiving rTMS and physiotherapy versus the group receiving physiotherapy regime alone. Of the 87 patients enrolled in this ongoing study, 19 were randomized to receive rTMS and 16 sham rTMS. All received physiotherapy. rTMS group received total 750 pulses @ 110% motor threshold (MT) with inter train interval of 45 seconds. Total duration per session was for 45 minutes. Between group analysis showed statistically significant improvements in the Study group with NIHSS, mBI, FMA lower limb post rTMS ($p< 0.05$) as compared to control group. No significant improvement in rTMS parameters MT ($p=0.15$), latency period ($p=0.11$) and MEP ($p=0.9$) was observed between groups. Serum VEGF of 20 patients was found to be statistically significantly elevated in the study group with a mean of 483.6±280.3 pg/ml as compared to controls ($p=0.04$). rTMS has proven to be a surrogate marker augmenting behavioural recovery after stroke [44].

Factors Influencing Outcome of Stem Cell Therapy Post Stroke

1) Types of stroke

Evidence obtained has been largely from intrastriatal implantation. Studies of the middle cerebral artery implantation rodent model have shown that the striatum is the primary site of damage and many believe that the resulting deficits in memory, learning and motor behavior are directly associated with striatal injury. Cortical lesions also may be accessible to transplantation, but infarcts involving white matter are more problematic. A proliferation of transplanted cells in the cortex may not necessarily repair underlying axonal damage. There is even rationale for neural transplantation in patients with pure white matter infarcts, which require an entirely different therapeutic strategy. The size and extent of infarction involving major arterial territories will play a significant role in patient selection. In patients with widespread damage, the number of cells potentially needed to restore function may be daunting [45,46].

2) When to infuse/transplant stem cells post stroke

The optimal time for intervention with stem cells post stroke remains unclear. In the acute setting, release of excitotoxic neurotransmitters, free radicals, proinflammatory mediators might threaten new tissue introduced into the peri-infarct region. Also, cells may be dying by apoptosis in the penumbra for several weeks after stroke. Inflammation leading to microglial activation may inhibit endogenous neurogenesis and may thereby suppress the growth and survival of transplanted cells.

In the acute stage, local repair processes are active, including the release of neurotrophic factors from the intrinsic milieu and the host environment during the early phase to facilitate implant growth, survival, differentiation and /or integration. The ischemic environment also promotes the generation of new neurons in periventricular regions and in the cerebral cortex. How transplantation will affect the on-going endogenous neurogenesis is unknown. There is accumulating evidence that stroke recovery involves plasticity of connections, which occur early after a stroke but may disappear months or years later. Transplantation might benefit from such plasticity and become maximally beneficial during this reorganization.

However, delaying the stem cell transplantation for several weeks after stroke must also contend with the disadvantage of formation of scar tissue which might adversely affect implanted cells. The choice of timing must also consider the natural course of recovery from stroke. Impairments have different courses of improvement depending on the type and severity. Many neurologists would therefore prefer to delay transplantation till the deficit plateaus. For these reasons and many others, some investigators have preferred to transplant at least a few months after a stroke. The two clinical trials have chosen to study disabled patients at least 6 months after a stroke. However, there are no corroborating animal models of chronic stroke. Few outcome measures exist for animals with chronic stroke infarcts. Most importantly, recovery in animals cannot be easily equated across studies or related to humans.

3) Blood supply

Transplantation is unlikely to succeed if there is a severe arterial occlusion without collateral circulation; inadequate blood supply would not support graft survival. In contrast, transplantation efforts in progressive degenerative disorders are not necessarily concerned with arterial patency and inflammation.

4) Site of implant

From a mechanical point of view, injection of cells into the fluid-filled cavity of a chronic infarct facilitates the migration of transplanted cells. Without a definable cavitated area, transplantation requires more direct pressure to inject risking damage to normal tissue. However, cavity fluid can dilute the concentration of donor cells.

In the acute setting, it may be appropriate to inject cells in the salvageable penumbra but grafts might still be exposed to the detrimental effects of spreading depression and excitatory neurotransmitters. Fetal cortical grafts to the ischemic brain have been shown to survive in the penumbra but not in the core lesion. However, in chronic infarcts, glial scarring might impede the delivery of cells to the penumbral areas.

Some investigators believe that grafts could be more effective if the poorly vascularized inflammatory environment of the ischemic region is avoided altogether and suggest the plausibility of transplantation to distant regions, even to the contra-lateral side.

5) Patient selection

Patients selected stem cell transplantation for stroke should have measurable deficits, impairments and handicaps. The neuroanatomical relationship between image-defined infarct and deficits should be well established. Co-morbidities and the need for extensive follow-up also play a strong role in determining which patients are good candidates for experimental therapies.

Evidence thus far

Animal studies

Stem/precursor cells from different sources have been tested for their ability to reconstruct the forebrain and improve function after transplantation in animals subjected to stroke [47-49].

The transplants, including a mouse neuroepithelial stem cell line, the human NTera-2 cell line, and human bone marrow cells, have been reported to partly reverse some behavioral deficits. However, in most cases, the underlying mechanisms are unclear and there is little evidence for neuronal replacement. Only few grafted cells have survived and they have not exhibited the phenotype of the dead neurons. Moreover, it is unknown if the observed grafted cells are functional neurons and establish connections with host neurons. Despite the poor evidence for significant neuronal replacement in these studies, improvement of various stroke-induced behavioral deficits has been observed. Stem cell transplantation probably can lead to clinically valuable improvements through several mechanisms. First, the tissue damage per se can stimulate plastic responses or interfere with neural activity in the host. Second, the transplants can act a biological minipumps and release a missing transmitter or secrete growth factors. These factors can stimulate plastic responses and improve the survival and function of host neurons. Third, the grafts can restore synaptic transmitter release by providing a local re-innervation. Fourth, and this is true neuronal replacement, the grafts can become integrated into existing neural and synaptic networks, and re-establish functional afferent and efferent connections.

Clinical Trials

NT2 neuron cell trials

Immortalized cell line NT2 is derived from a human testicular germ cell tumor more than 20 years ago. Unlike other teratocarcinoma cell lines, the NT2 cells show an exclusive commitment to a neural lineage when exposed to retinoic acid. Several studies have shown that NT2 cells resemble neural stem cells. They express cell surface markers and cytoskeletal proteins unique to neural stem cells. Treatment with retinoic acid and mitotic inhibitors for several weeks ultimately results in the production of postmitotic, NT2N, which expresses neurotransmitters, functional neurofilament and cytoskeletal proteins, and other proteins indicative of secretary activity and synaptogenesis. Transplanted cells also release neurotransmitters and elaborate typical neuronal proteins [50,51].

Phase I: Seven years ago, a clinical trial began to assess the safety of intrastriatal NT2N (produced by Layton Bioscience Inc. and known as LBS neurons for human use) transplantation in patients with basal ganglia infarcts and stable motor deficits 6 months to 6 years before transplantation. Twelve patients were treated with NT2N cell transplants and immunosuppressed using cyclosporine for 9 weeks. Based on preclinical safety data, doses of 2 and 6 million cells were considered appropriate. Four years after the study began, there have been on adverse events related to the implants. Two patients died of unrelated medical illnesses. On autopsy examination of one of these patients, who did not show clinical improvement and died of myocardial infarction, the graft site showed no signs of inflammation, neoplasia or infectious disease 27 months after implantation. Because NT2N cells are polyploidy for chromosome 21, grafted neurons were identified at the injection site with fluorescent in situ hybridization and DNA probes specific to this distinctive chromosomal feature. Positron emission tomography (PET) scanning at 6 months showed greater than 15% relative uptake of F-18 flourodeoxyglucose at the transplant site in six patients. This may reflect surviving and functioning implanted cells, enhanced host cell activity or an inflammatory response.

Phase 2: A randomized open-label trial with observer blinded neurological evaluations was undertaken to test the effectiveness of neuronal cell transplantation in patients with substantial functional motor deficits following basal ganglia infarction. Fourteen patients were randomized to receive 5 or 10 million implanted cells followed by rehabilitation, compared with 4 patients who only underwent physiotherapy. Patients had stable motor deficits 1-6 years after the onset of stroke. Half the patients had an ischemic stroke, and the other half had a hemorrhage. The author tested the hypothesis that implantation of neuronal cells would be safe, feasible and improve motor neurologic deficits. One patient had a single seizure and another had a subdural hematoma evacuated 1 month after transplantation. There were no cell-associated adverse events [52,53].

Functional outcomes were assessed by the National Institutes of Health Stroke Scale (NIHSS), European Stroke Scale Score, Stroke Impact Scale, Fugel-Meyer Score, and Action Research Arm testing. Cognition was also tested before treatment and after 6 months. Transplant patients showed a trend toward improvement in functional outcomes on several scales compared with baseline measurements before transplantation, but there were no statistically significant trends compared with the four controls. With such small numbers however, the significance of the findings is unclear. A third clinical trial will evaluate cell implantation for patients with stable cortical strokes.

Diacrin trial

Phase I: A pilot safety and feasibility study was started in 1998. The original goal was to enroll 12 patients with chronic, stable, moderate-sized basal ganglia infarcts who would receive intrastriatal implantation of fetal cells from the porcine, primordial striatum, also called the LGE of porcine embryonic tissue and pretreated in culture with an anti-major histocompatability complex class I antibody, thus obviating the need for immunosuppression after transplantation. Five patients underwent transplantation. Their strokes occurred on an average five years earlier. Computed tomography at the completion of surgery showed no evidence of hemorrhage in any patient. The patients developed no new neurological deficits in the acute setting. One patient developed cortical vein occlusion thought to be related to the surgery, but the Food and Drug Administration terminated the study. At 2 years one of the patients showed improvement on the modified Rankin Scale [54,55].

The korean university trial

This study was completed in 2005. It was a randomized controlled phase I/II trial. Cell transplantation improved recovery from ischemic stroke in 30 patients with intravenous autologous mesenchymal stem cells infusion .They prospectively and randomly allocated 30 patients with cerebral infarcts with middle cerebral artery territory and with severe neurological deficits into two treatment groups : the MSC group (n=5) received intravenous infusion of $1x10^8$ autologous MSCs whereas the control group (n=25) did not receive MSCs. MSC treated patients received $5x10^7$ cells twice: 4 to 5 (first boosting) and 7 to 9 weeks (second boosting) over 15-20 minutes. Neurological deficits and

improvements in function were compared between the groups for 1 year after symptom onset. Neuroimaging was performed serially in five patients from each group. Outcomes improved with the MSC treated patients compared with the control group [56].

Adult Stem Cell Therapy in Stroke

Adult stem cell therapy for stroke can be divided in an endogenous and exogenous approach. The aim of the endogenous stem cell therapy is to exploit the population of adult stem cells already physiologically present either in the CNS or hematopoetic system derived adult stem or precursor cells are administered locally or systemically after purification and propagation in culture [57,58].

Interestingly, acute cerebral ischemia in human individuals leads spontaneously to a threefold increase in CD34+ cell count in the peripheral blood. Considering this change as an insufficient self-repair mechanism, it is a logical consequence to further promote CD34+ cell mobilization pharmacologically by the administration of granulocyte colony stimulating factor (G-CSF). In addition, G-CSF has been described to exert neuroprotective effects following cerebral ischemia. A recent preclinical study found functional improvement in rats with focal G-CSF. There are ongoing clinical studies with G-CSF in acute ischemic stroke [59].

Clinical Trials on Autologous Bone Marrow derived Stem Cells Therapy in Chronic Stroke from India

This research dealt with the safety and efficacy of intravenous autologous bone marrow derived mononuclear and culture expanded mesenchymal stem cells in stroke. Adult patients were recruited with the inclusion criteria as: 3 months to 2 years after stroke, power of hand muscles of at least 2; Brunnstrom stage 2-5; NIHSS of 4-15, conscious and cooperative. This was an unblinded, non randomized case control study. Patients were assessed for strength, tone (modified Ashworth), Fugl Meyer (FM) scale for upper limb, Edinburgh handedness inventory, modified Barthel Index (mBI) and functional MRI including DTI was performed at baseline, 8 and 24 weeks of stem cell infusion. Prior to stem cell therapy, patients were screened and educated about stem cells and bone marrow aspiration technique. Forty stroke patients were recruited with the above inclusion criteria. Twenty were given stem cells followed by 8 weeks of physiotherapy, serving as experimental/stem cell group and 20 patients were administered physiotherapy regime alone. 50 -60 million cells in 250 ml of saline was infused intravenously over 2-3 hours. The baseline clinical and radiological scores between the experimental and control groups were statistically insignificant. The safety profile was normal with no mortality or cell related adverse reactions in stem cell patients. On comparison between experimental and control groups, mBI was statistically significant on follow up at 24 weeks ($p = 0.05$). Laterality Index (LI) of BA 4 and BA 6 was insignificant at 8 and 24 weeks follow up, as also in the FA ratio, fiber length and fiber number ratio between the two groups. An increased number of cluster activation in Brodmann areas BA 4, BA 6 was observed post stem cell infusion indicating neural plasticity. The study concluded that autologous intravenous stem cell therapy is safe and feasible. Stem cells may act as "scaffolds" for neural transplantation and may aid in repair mechanism [60-62].

There are many factors which still need to be investigated in preclinical and clinical designs of stroke models such as tagging of cells intravenously with super paramagnetic iron oxide (SPIO) particles to study the homing in mechanism by stem cells. *In vivo* monitoring of stem cells after grafting is essential for the follow up of their migrational dynamics and differentiation process. ReNeuron Group, a UK based stem cell therapy business applied for Investigational New Drug (IND) proposal to FDA to commence stem cell trial with Re N001 stem cell line. This cell line is conditionally immortalized using fusion transgene c-mycER to allow controlled expansion when cultured in the presence of 4-hydroxytamoxifen. Recently IND has been put on hold in clinical trials. Currently two other clinical studies are recruiting patients for autologous human stem cells transplantation in stroke (PISCES). The area of stem cells research is vast and can be explored in various ways in stroke. Since the previous studies have used a small sample size and owing to the safety concerns, large scale clinical trials with long term follow up are needed. Numerous fundamental questions (inclusion criteria, patient's age, type of stroke, cell type, dose of cells, route and site of delivery etc) need to answered by more randomized controlled trials [63,64].

Pharmacotherapy for Neurorestoration

Role of NO

Nitric Oxide (NO) received attention when it was discovered that "endothelial-derived relaxing factor" was NO, an integral molecule involved with maintaining endothelial cell integrity, as well as participating in hemodynamic homeostasis. A variety of cells, including vascular smooth muscle cells and neurons, produce NO either constitutively or inducibly following perturbation. The increased expression of neuronal NO synthase within the subvenricular zone (SVZ) during embryogenesis suggests a role for the NO pathway in neurogenesis [65]. The administration of NO donors increases neurogenesis in the adult rat SVZ and dentate gyrus, suggesting an expanded role for the NO cascade beyond embryogenesis. Treatment with NO donors beginning 24 hours post stroke in rat models is associated with increased neurogenesis and improvement in functional outcome despite no change in infarct volume. NO is also a potent activator of soluble guanylate cyclase, the enzyme that converts GTP to cGMP. Thus the delivery of an NO donor increases cGMP levels within both ischemic and non-ischemic rat brains, suggesting a permissive role for NO in neurogeneisis and that cGMP may serve at least in part as a downstream mediator of the NO effects. In addition to enhancing cGMP levels by augmenting NO availability, cGMP levels may also be increased by inhibiting its metabolism by the Phosphodiesterase 5 (PDE5) enzyme. The strategy of increasing the downstream mediator cGMP without affecting NO levels may be preferred due to the mixed outcomes in stroke reported in animal models following alterations in NO levels [66]. A major PDE5 inhibitor is sildenafil. Animals treated with sildenafil post stroke achieved significant and substantial increase in neurological functional recovery. Phase I trials in humans with acute stroke are currently on going. Sildenafil demonstrated improved cerebral blood flow (CBF), neurogenesis, angiogenesis, and synaptogenesis following experimental stroke, even when therapy is delayed for up to 1 week and aged animals are examined. In these studies, the improvements in functional outcome that occur despite no change in infract volume are intriguing [67]. Ultimately, functional improvements that are robust and persistent will need to be demonstrated in clinical trials. Surrogate markers that may assist in answering these questions include functional MRI or diffusion tensor imaging (DTI), which can demonstrate improvements in structure, organization and functional connectivity.

Statins

Other agents which also such as statins, also greatly enhance neurological recovery post stroke [68]. Drugs which increase high density lipoproteins (HDL) such as slow release niacin have also been employed to treat stroke and have shown substantial neurological benefit when treatment is initiated days after stroke. Other neurorestorative agents under investigation are erythropoietin (EPO), carbamylated EPO (CEPO), and Thymosin B4.

Role of GABA

Recovery after stroke involves remapping of the neuronal circuitry in the regions adjacent to the site of injury or the peri infarct zone. A pharmacological approach to re-establish functional neuronal connections that are lost during stroke could enhance current physical rehabilitation therapies. Recently, Clarkson et al showed that inhibiting tonic GABA (gamma amino butyric acid) ergic signaling days after stroke can improve locomotor function, suggesting a therapeutic approach that is less time sensitive than acute reperfusion therapies. ABA signaling reduces neuronal excitability and thereby modulates synaptic plasticity [69].

Minocycline as a Putative Agent for Restorative Therapy Post Stroke

Minocycline is the second generation tetracycline derivative known to have anti-inflammatory effects independent of its antimicrobial action [70]. Recent studies have shown that minocycline prevents microglial activation, and also has notable beneficial effects in animal models of global and transient focal cerebral ischemia and other brain injuries. The proposed mechanisms of minocycline include anti-inflammatory effects, reduction of microglial activation, MMP reduction, nitric oxide production and inhibition of apoptotic cell death [71]. In a randomized single blinded study, Padma et al studied the effects of oral Minocycline (200 mg/day for 5 days) post stroke versus placebo. Of 50 patients included in the trial, patients who received minocycline had significant improvement in stroke outcome as noted on NIHSS, mBI and mRS scores [72]. Larger trials are needed to confirm the above findings.

Others

Recombinant erythropoietin (Epo) was reported to be safe and efficacious in a proof-of-concept study [73]. A phase II/III study (522 patients) was negative and showed a higher death rate and complications in patients receiving Epo; possible interaction with rTPA was cited as a likely cause of increased mortality. Intravenous granulocyte colony stimulating factor (G-CSF) has also been investigated ina dose escalation phase IIa study (AXIS: 44 subjects, drug administered within 12 hours). The authors reported good tolerability and suggest further investigation [74,75].

Cerebrolysin, a peptide-based drug is another candidate with potential for approval to be used as a restorative agent. Multiple laboratories have demonstrated the safety and efficacy of this drug in the treatment of experimental stroke. Cerebrolysin is presently in clinical trials and is in use in some countries for the clinical treatment of stroke. Cerebrolysin is seen on induce neurogenesis and angiogenesis in animal models of stroke and concomitantly enhances brain plasticity and recovery from stroke.

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