Stem cell therapies in preclinical models of stroke. Is the aged brain microenvironment refractory to cell therapy?

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Highlights

- The aged rat brain microenvironment is not necessarily refractory to cell survival and may support cell survival and angiogenesis
- It is not clear if the transplanted cells have any beneficial effect on behavioral recovery
- The efficacy of cell therapy can be enhanced by physical rehabilitation
- There remain significant developmental and translational issues that remain to be resolved

ABSTRACT

Stroke is a devastating disease demanding vigorous search for new therapies. Initial enthusiasm to stimulate restorative processes in the ischemic brain by means of cell-based therapies has meanwhile converted into a more balanced view recognizing impediments that may be related to unfavorable age-associated environments. Recent results using a variety of drug, cell therapy or combination thereof suggest that, (i) treatment with Granulocyte-Colony Stimulating Factor (G-CSF) in aged rats has primarily a beneficial effect on functional outcome most likely via supportive cellular processes such as neurogenesis; (ii) the combination therapy, G-CSF with mesenchymal cells (G-CSF + BM-MSC or G-CSF + BM-MNC) did not further improve behavioral indices, neurogenesis or infarct volume as compared to G-CSF alone in aged animals; (iii) better results with regard to integration of transplanted cells in the aged rat environment have been obtained using iPS of human origin; (iv) mesenchymal cells may be used as drug carriers for the aged post-stroke brains. Conclusion: While the middle aged brain does not seem to impair drug and cell therapies, in a real clinical practice involving older post-stroke patients, successful regenerative therapies would have to be carried out for a much longer time.

AGE AS A KEY FACTOR IN PRECLINICAL STROKE STUDIES

Cerebral ischemia is a common disease in the older population and the second most common cause of death in Europe, and the third leading cause of death in Canada and the United States (Lloyd-Jones, et al., 2010; Roger, et al., 2012).

Age is the principal nonmodifiable risk factor for cerebral ischemia. The incidence of stroke increases significantly with age in both men and women, with half of all strokes occurring in people over the age of 75, and one-third in the population over age 85 (Roger, et al., 2012). Further, there are gender differences in the incidence of stroke by age subgroups. The incidence of stroke is higher in men up to age 75, similar in the 75-84 age group, and higher in women in the age group older than 85 (Roger, et al., 2012). This may be attributed to the longer life span in women. There are many other gender differences with regard to stroke outcome, risk factors, treatment, and mortality that have more complicated and unexplained underlying etiologies. The age-associated decline in functional reserves is most pronounced in advanced age of 85 or older and implys an impaired response to stressors and illnesses.

Overall, age-associated changes also show great variability among individuals and are often impacted by genetic and long-term lifestyle factors (Wolfson, et al., 2009; Tacutu, et al., 2010; Tacutu, et al., 2011).

STROKE MODELS USING AGED ANIMALS ARE CLINICALLY MORE RELEVANT

Studies of stroke have demonstrated an age and gender effects on incidence, functional recovery and mortality, not only in humans but also in animal models (Bergerat, et al., 2011; Gokcay, et al., 2011). Indeed, the age-dependent increase in the evolution of ischemic tissue into infarction strongly suggests that age is a biological marker for the variability in tissue

outcome in acute human stroke (Ay, et al., 2005).

Over the past 20 years, suitable models for stroke in aged rats have been established. All are based on the middle cerebral artery occlusion (MCAO). MCAO has been produced with permanent or transient occlusion for 30-120 min using a thrombus through intraluminal filament occlusion or a hook attached to a micromanipulator, or by occlusion of distal branches of the MCA, while long-term hypoxia-ischemia could also be induced by unilateral common carotid artery occlusion (reviewed in Popa-Wagner et al., Frontiers Cell Neuroscience 2014).

Since epidemiological studies have shown that human stroke occurs more often in late middle age (50-70 years) than in older subjects (over 70 years) (Feigin, et al., 2003) justify the use of stroke models in middle aged animals (Popa-Wagner, et al., 2007).

LIMITED BENEFICIAL EFFECTS ON MORTALITY, BEHAVIOURAL RECOVERY AND NEUROGENESIS OF G-CSF IN AGED RATS

The hematopoietic factor Granulocyte-Colony Stimulating Factor (G-CSF), has been shown to reduce infarct volume and improves behavioral outcome after various types of experimental stroke (Shyu, et al., 2004; Lee, et al., 2005; Xiao, et al., 2007; Han, et al., 2008). Under ischemic conditions, G-CSF inhibits programmed neuronal cell death (Komine-Kobayashi, et al., 2006) and stimulates neural progenitor cell differentiation. These mechanisms and others, including immunomodulation and blood vessel plasticity, are currently thought to be responsible for infarct size reduction and improved functional outcome in young-adult rodent stroke models (Minnerup, et al., 2008).

One potential weakness of the preclinical dataset is, however, the lack of proof in aged subjects. It is in fact a general drawback of preclinical evaluations of candidate stroke

drugs that due to cost effectiveness and practicability most studies were done in young animals. A lack of data from aged subjects in preclinical studies may at least in part explain the failure of candidate neuroprotective drugs in clinical trials. The aged brain has compared to the young brain an enhanced susceptibility to stroke and displays a limited recovery from an ischemic injury (Popa-Wagner, et al., 1998; Badan, et al., 2003; Rosen, et al., 2005). Therefore we assessed the treatment effects of G-CSF on mortality, behavioral function, infarct volume, and neurogenesis in 19 to 20 month old male Sprague-Dawley rats subjected to 90 min occlusion of the middle cerebral artery (MCA).

One of the remarkable effects of G-CSF treatment was a significant decrease in the mortality rate. However, there was no significant effect of G-CSF on reducing infarct volumes. In contrast to young animals - where recovery of function is complete even after short terms of G-CSF treatment - functional recovery of motor function (rotarod, inclined plane) in aged animals occurred predominantly during the treatment period and was therefore limited to the first 12 days after stroke onset, except the radial maze the beneficial where the beneficial effect lasted for 21 days. G-CSF treatment also had a beneficial effect on functional recovery of motor function (rotarod, inclined plane) and working memory (radial maze). However, the beneficial effect of treatment was generally limited to the first 12 days post-stroke. A stereological analysis of the number of BrdU labeled cells in the SVZ revealed a significant increase in the number of proliferating cells in G-CSF treated animals compared to vehicle treated animals. Further, the G-CSF treatment increased the number of proliferating cells in the SVZ and the dentate gyrus and increased the number of new born neurons in the SVZ, ipsilateral to the lesion (Fig. 1).

Cell therapy of stroke using mesenchymal stem cells

Cellular therapy (Fig. 2) can enhance the endogenous restorative mechanisms of the injured brain by supporting processes of neovascularization, neurogenesis, neural reorganization and functional recovery (Chen, et al., 2005; Crigler, et al., 2006; Bao, et al., 2011; Lim, et al., 2011; Hayase, et al., 2009; Hsieh, et al., 2013).

Mesenchymal stromal cells (MSCs), derived either from bone marrow or from adipose tissue, have been shown to ameliorate the clinical outcome in experimental model of cerebral ischemia (Kocsis, et al., 2012; Honmou, et al., 2012). Administration of MSCs in acute stroke animal models markedly decreased brain infarct size, improved neurological function by enhancing neurogenesis, and showed anti-inflammatory and antiapoptotic effects. Additionally, initial clinical studies using intravenously delivered MSCs have been initiated in human subjects with stroke (Lee, et al., 2010).

It has been shown that MSCs are able to release several angiogenic and neurotrophic factors, as well as anti-inflammatory molecules when localized in an inflammatory microenvironment *in vivo* (Dharmasaroja, 2009; Blasi, et al., 2011). In addition, MSCs seem to have a good capacity to home to ischemic brain when administrated through a systemic route (Gutiérrez-Fernández, et al., 2013).

Several studies showed that grafting of bone marrow-derived stem cells in the peripheral circulation improved functional neurological outcome and reduced infarct volume (Honmou, et al., 2012). Most of these studies used bone marrow mesenchymal cells (BM MSC) but feasibilty and safety in clinical trials was also shown for the bone marrow mononuclear cells (BM MNC) (Hermann, et al., 2012; Moniche, et al., 2012). A conclusive result on optimal timing and dosing is, however, still missing.

COMBINATION THERAPIES OF STROKE

Cellular therapy using mesenchymal stem cells(MSC) can enhance the endogenous restorative mechanisms of the injured brain by supporting processes of neovascularization, neurogenesis, and neural reorganization (Hayase, et al., 2009; Bao, et al., 2011; Lim, et al., 2011; Hsieh, et al., 2013). In addition, the outcome was improved in MSC-treated patients with severe cerebral infarcts (Bang, et al., 2005).

Recently we could show for the first time, that G-CSF treatment in aged rats after stroke enhances survival, functional neurological recovery, and enhances neurogenesis (Popa-Wagner, et al., 2010). Next, we reasoned that the efficiency of the bone marrow derived-cell therapy may be increased by simultaneous application of G-CSF. In particular we tested the hypothesis that grafting of pre-differentiated human mesenchymal cells (BM MSC) in G-CSF-treated animals improves long term functional outcome in aged rodents (Balseanu, et al., 2014).

The post-stroke therapy with the combination of BM-MSC with G-CSF started early, at 6 hrs after stroke. In the cortical model of stroke, therapeutic cells injected via the jugular vein probably enter the injured brain via the lateral ventricle as shown by the human CD166-positive cells. A fraction (about 1%) of the injected CD166- and CD105-positive cells reached the infarcted area where they were intermingled with surviving or degenerating neuronal nuclei. Noteworthy was also the presence of immunopositivity for human nuclei that were dispersed between the rat nuclei in the infarcted area. In the ipsilateral hemisphere, the injected human BMSCs were detected in the corpus callosum as shown for CD166-positive cells and CD105-positive cells (Balseanu, et al., 2014).

The mechanisms by which MSCs may ameliorate infarcted brain tissue seem related more to the capacity of MSCs to release neuroprotective factors (paracrine mechanism) than

to their capacity to replace damaged neural cells through their transdifferentiation properties. Thus, beyond the formerly infarct core, several groups noted vigorous sprouting angiogenesis as evidenced by RECA/BrdU double positive immunostaining of the blood vessels as well as numerous $BrdU^+$ nuclei in the newly formed endothelium and reconstruction of the basal lamina during the resolution phase of angiogenesis. By number of laminin/BrdU co-localizations, the density of the newly formed blood vessels was significantly higher in the brains of animals treated with the combination G-CSF + BM-MSC as compared to controls and G-CSF alone (Balseanu, et al., 2014).

These results strongly suggest that the BM MSC promoted angiogenesis rather neurogenesis in the injured area (Bronckaers, et al., 2014). Indeed, previous studies have shown that delayed intracerebral injection of hMSCs modified the cerebral microvasculature after transient ischemia (Moisan, et al., 2012) and improved the cerebral blood flow (CBF) (Jiang, et al., 2012).

MESENCHYMAL CELLS CAN BE USED AS DRUG CARRIERS FOR STROKE THERAPY

Cyclin-dependent kinase-5 (Cdk5) is over-expressed in both neurons and microvessels in hypoxic regions of stroke tissue and has a significant pathological role following hyper-phosphorylation leading to calpain-induced cell death. Recently, the neuronal cyto-protective potential of a natural small peptide (CIP-peptide) was demonstrated after neurotoxic stress. CIP is a derived-p35 cleavage peptide, which selectively targets Cdk5/p25 activity without affecting Cdk5/p35 signalling. In hypoxia, insertion of Cdk5/p25-inhibitory peptide (CIP) vector preserved and enhanced *in vitro* angiogenesis (Bosutti, et al., 2013). Indeed, intracortical administration of mesenchymal harboring the CIP-peptide to middle

aged rats with stroke increased survival of transplanted cells in th perinfarct area as shown by double immunofluorescence (Fig. 3).

CELL THERAPY OF STROKE USING INDUCIBLE PLURIPOTENT CELLS

Recent studies indicate that inducible pluripotent cells (iPSCs) can also be generated from aged humans and differentiate into specific cell types (Mohamad, et al., 2013; Phanthong, et al., 2013). Moreover it seems that the re-differentiation efficiency of human fibroblasts via iPSCs into functional motor neurons is the same as in 29-82 years old individuals (Boulting, et al., 2011). However, if and how the aged brain responds to grafted iPSCs is largely unknown.

In stroke models, hiPSC-lt-NES cells derived from a young adult male have the potential to survive, differentiate into immature and mature neurons, and migrate to the peri-infarct area of aged rats. The treated aged rats showed improved behavioral recovery after implantation into the stroke-injured striatum and cortex of adult rats (Oki, et al., 2012; Tornero, et al., 2013). Likewise, transplantation of hiPSC-lt-NES cells into the perilesional cortex after stroke in aged rats reduces the number of dead neurons (Fig. 4). Grafts of human iPSC-lt-NES cells also diminished the number of activated microglia/macrophages in stroke-damaged cortex of aged rats (Tatarishvili, et al., 2014).

Conclusions

Therapy of stroke with both BM MSC and NPCS suggest that the aged rat brain microenvironment is not necessarily refractory to cell survival and may support cell survival and angiogenesis. However, it is not clear if the transplanted cells have any beneficial effect on behavioral recovery.There remain significant developmental and translational issues that remain to be resolved in future studies such as (i) understanding the differentiation into

specific phenotypes (Svendsen, 1997; Saporta, et al., 2001); (ii) tumorigenesis remains a significant concern (Roy, et al., 2006; Riess, et al., 2007); (iii) anti-neuroinflammatory therapies are a potential target to promote regeneration and repair in diverse injury and neurodegenerative conditions by stem cell therapy; (iv) finally, the efficacy of cell therapy can be enhanced by physical rehabilitation (Dunnett, 2013).

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Legends to figures

Figure 1. Three-dimensional imaging of several colocalized BrdU (red)/NeuN (green) cells in the SVZ of the infarcted hemisphere.

Figure 2. Stem cell therapy of stroke. (**A**) Old rat with striatal infarct; (**B**) Documentation of the striatal infarct by MRI; (**C**) Injection of NPCs into lesioned striatum; (**D**) build up of an hypothetical neuronal network weeks later; (E) Behavioural assessment of cell treatment.

Figure 3. Intracortical administration of mesenchymal harboring the CIP-peptide to middle aged rats with stroke increased survival of transplanted cells in th perinfarct area as shown by double immunofluorescence. HuNu (green) (**A, D**) stains the exogenous mesenchymal cells used as carriers; NeuN (red) was a marker of endogenous cells (**B**). CD105 is another marker of mesenchymal cells (**C**).

Figure 4. Human iPSC-lt-NES cells survive transplantation into stroke-damaged cortex of aged rats, stop proliferation, and differentiate into neurons. Fluorescence microscopic and confocal images of hiPS-lt-NES cell-derived HuNu+ cells co-expressing neuroblast marker DCX (**A**), and GABA (**B**) marker at 8 weeks after transplantation.