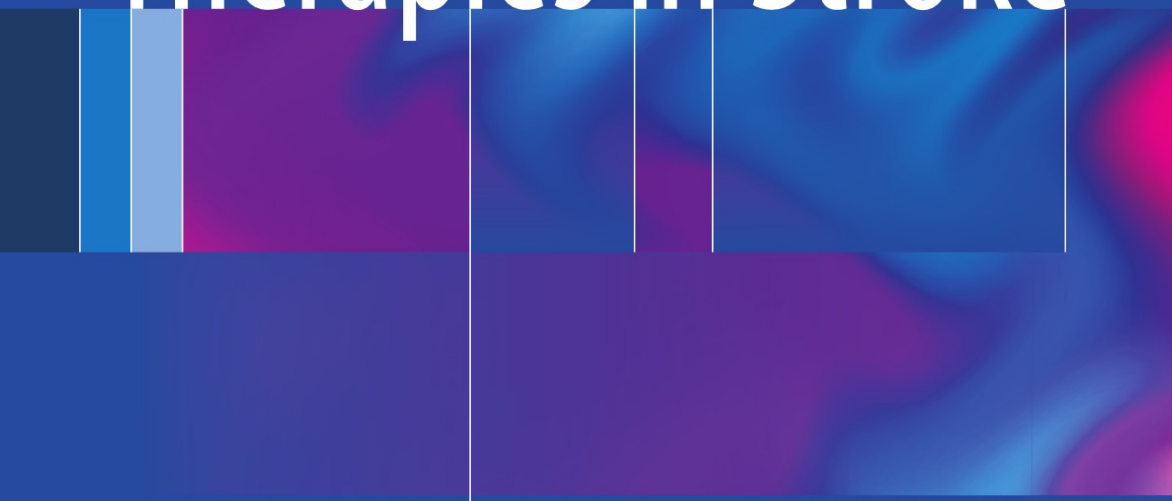
Jukka Jolkkonen
Piotr Walczak *Editors*

Cell-Based Therapies in Stroke



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Preface

Stroke is a major cause of severe disability that poses an enormous healthcare burden. The only effective therapy is thrombolysis when started within 4.5 h of symptoms onset, but, because of the narrow therapeutic time window, only few patients benefit from it. Despite extensive research, neuroprotective drugs have all failed in clinical trials. Thus, efforts focused on the pursuit of new cell-based restorative therapies have emerged recently as attractive approaches for the treatment of stroke patients.

Indeed, the preclinical data regarding cell transplantation show great potential. However, in light of so many failed neuroprotective strategies, these promising results should be evaluated vigilantly before clinical translation. Cell-based therapy research is now moving to early phase safety and feasibility studies in stroke patients, very cautiously, to pursue the translation of experimental data to clinical practice. At the same time, researchers are under tremendous pressure from investors, funding bodies, and patients who require faster progress in research and expect immediate results in the clinical realm. Another unfortunate phenomenon we currently face is that private clinics worldwide offer cell transplantation without hard evidence of its efficacy or even safety.

In this book, we provide a critical update on cell-based therapies, covering a broad range of topics from experimental studies to very early phase clinical trials. The adult brain is not as rigid as once thought, and several repair mechanisms are activated in response to injury, including angiogenesis, neurogenesis, and synaptogenesis. In the first chapter, Gary Steinberg and his colleagues explore the mechanisms through which transplanted cells may facilitate functional recovery. Allison Willing and her colleagues, in the second chapter, discuss various cell delivery routes. Both intraparenchymal transplantation and systemic delivery of cells have been applied, but the most effective route is still unknown. Surprisingly, it seems that cells may not even have to enter the brain to act. A variety of cell preparations are under active investigation and, regardless of source, cells seem to improve behavioral performance in experimental models. Cesario Borlongan, in Chap. 3, provides a review of the current knowledge of different cell sources and their benefits and limitations. In Chap. 4, Dale Corbett and his coworkers assess variables such as environmental enrichment that mimics rehabilitation after stroke and how these variables might affect the efficacy of cell treatment and other restorative therapies.

The subsequent chapters focus on the use of translational imaging modalities as tools that facilitate the progress of cell-based therapies. Jeff Bulte, in Chap. 5, explores novel cell labeling techniques for MRI studies. In the following chapters, Piotr Walczak and Mike Modo describe how real-time MRI can be applied to guide cell transplantation, including optimizing cell delivery and targeting, as well as monitoring tissue regeneration. Johannes Boltze and coworkers, in Chap. 8, describe autologous cell homing after intravenous infusion in a unique large animal model (gyrencephalic brain), which closely simulates clinical conditions. Raphael Guzman and coworkers describe X-ray fluorescence imaging that enables high-resolution views and iron quantification within individual cells. In Chap. 10, Jukka Jolkkonen and coworkers explore the use of SPECT in whole-body biodistribution studies. SPECT is truly a translational imaging modality as the same tracers can be used in animals and patients.

The final four chapters provide an insight into early phase studies in stroke patients. Douglas Kondziolka and coworkers review completed trials of intracerebral cell transplantation in stroke patients, which show feasibility and safety. In Chap. 12, Sean Savitz and coworkers explore intravenous cell delivery as the least invasive cell delivery route in stroke patients. In Chap. 13, Gabriel de Freitas and Rosalia Mendez-Otero summarize pilot studies using intra-arterial delivery of cells in stroke patients. Lastly, Miroslaw Janowski describes a few rare patient cases where cells or a growth factor were delivered via the intracerebroventricular route.

We hope this volume will help readers understand the limitations and advantages of cell-based therapies in stroke and will minimize the risk of translational failures or premature interruption of challenging clinical trials designed to treat this devastating disease.

Jukka Jolkkonen
Piotr Walczak

Contents

Part I

Preclinical Optimization of Cell Therapies

Cell Therapy and Structural Plasticity Following Cerebral Ischemia

1

Stanley Hoang, Henry Jung, Tonya Bliss,
and Gary Steinberg

1.1 Introduction

Stroke is one of the leading causes of adult disability in the world. For ischemic stroke, the main therapy is the clot lytic agent tissue plasminogen activator, which must be administered within the first 4.5 h (Del Zoppo et al. 2009). As this time frame is limited and depends on the acute detection of clinical symptoms, much research has focused on preventing secondary injury after an initial ischemic or thrombotic event, although clinical translation has been limited (Ginsberg 2008). A new paradigm shift in therapeutic targets for stroke focuses on brain repair, particularly the brain's plasticity—the ability to regenerate synaptic structures and reorganize its functional architecture after injury. This phenomenon is observed in many stroke patients who often initially present with acute loss of motor function but then regain a remarkable degree of functional independence in the weeks or months following the insult. Experimental studies have shown that the surrounding uninjured parenchyma generates new dendrites and axons that can, to some extent, compensate for the functional loss of injured tissue.

Since brain plasticity occurs days to months after stroke, therapies that target such brain repair would significantly open the therapeutic time window of intervention, thus benefiting a significantly larger patient population than current strategies. Growing evidence points to the potential of cell transplantation therapy to promote

Stanley Hoang and Henry Jung contributed equally to this work

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brain plasticity after stroke as will be discussed in this chapter. An assortment of cell types, including brain-, bone marrow-, blood-, and dental pulp-derived progenitors, enhance recovery in experimental models of stroke (Chen et al. 2001a; Darsalia et al. 2011; Guzman et al. 2008a; Hicks et al. 2009; Kelly et al. 2004), reviewed in Bliss et al. (2007), and hemorrhagic stroke (reviewed in Andres et al. 2008b). Due to these early auspicious results, phase I and II clinical trials are now in progress (Andres et al. 2008a; Locatelli et al. 2009; Onteniente and Polentes 2011; Wechsler 2009) where a positive outcome will bring us closer to a much needed new therapeutic strategy for stroke. Understanding the mechanism of action of the transplanted cells will facilitate the successful translation of cell transplantation strategies to the clinic. Here we discuss the effects of transplanted cells on brain plasticity after stroke.

1.2 Anatomical Reorganization as the Basis of Functional Recovery After Stroke

Following an ischemic or hemorrhagic stroke, the immediate devastating neurological deficits on motor and cognitive abilities usually improve within the first few weeks and gradually into the first year (Benowitz and Carmichael 2010). The initial recovery is attributed to the reduction in edema and the inflammatory response. Long-term recovery, however, is most likely due to plastic mechanisms at the synaptic levels that lead to the reorganization of the ischemic penumbra (Benowitz and Carmichael 2010; Dancause 2006; Murphy and Corbett 2009). In humans, functional imaging has revealed compensatory recruitment of areas ipsilateral and contralateral to the stroke site during cognitive tasks (Cramer 2008). This activation pattern becomes more refined to ipsilateral cortical areas with time and correlates with good recovery. Similar results are seen with animal studies (Dijkhuizen et al. 2003; Takatsuru et al. 2009), which suggest this gross remapping is caused by local and long distant changes in axonal sprouting and dendritic arborization (Gonzalez and Kolb 2003; Jones and Schallert 1992).

1.3 Dendritic and Axonal Reorganization After Stroke

Many studies provide evidence for remodeling of dendrites in the ischemic penumbra surrounding the cortical infarct. Chronic changes in dendritic structural plasticity after stroke have been reported with increased contralesional layer V dendritic branching peaking at 18 days post stroke (Jones and Schallert 1992), while ipsilesional layer III branching was decreased (compared with uninjured animals) at 9 weeks post stroke (Gonzalez and Kolb 2003). Exposing animals to an enriched environment after ischemia increased dendritic complexity in the contralateral cortex and enhanced functional recovery (Biernaskie and Corbett 2001), although a causal link between the two was not proven. Brown et al. showed an increase in dendritic spine density and turnover rates in the penumbra area close to the stroke region in the first 2 weeks after stroke (Brown et al. 2009). Interestingly, the increase

in spine density was greatly affected by activity frequency, where restriction of limb use leads to less dendritic sprouting and performance of complex tasks results in significant complex sprouting (Jones et al. 1999).

Axonal regeneration has likewise been demonstrated in different models of ischemic stroke with new projections thought to target areas denervated by the stroke injury (Benowitz and Carmichael 2010). In rodent and primate models of ischemic cortical injury, such sprouting has been observed locally around the infarct area (Carmichael et al. 2001; Conner et al. 2005; Dancause et al. 2005; Li et al. 2010). For example, the rat barrel cortex following an experimental stroke has shown a robust axonal sprouting response that remaps the body representation of the somatosensory cortex (Carmichael et al. 2001). For this experiment, the axonal tracer biotinylated dextran amine (BDA) was administered into the rat whisker barrel (somatosensory) cortex bordering the infarct region 3 weeks after focal strokes revealing new intracortical projections that arose from the ischemic penumbra. In primates after brain injury, the primary motor area also undergoes significant axonal reorganization with circuits of the premotor and somatosensory areas, thus demonstrating that stroke induces axonal sprouting near the injury site and also promotes novel connections with areas distant from the injury (Dancause et al. 2005). In addition to axonal sprouting in the injured cortex, interhemispheric axonal outgrowth from the intact cortex to the injured hemisphere has also been observed after stroke (Carmichael 2008). Moreover, long descending pathways from the cortex to the spinal cord, such as the corticospinal tract that mediates voluntary movements, also reorganize in association with the recovery of limb function in rodents (Liu et al. 2008; Weidner et al. 2001), often with cross midline sprouting from the intact spinal cord to the denervated areas of the injured spinal cord (Chen et al. 2002). Despite measurable brain plasticity after stroke, axonal and dendritic reorganization is limited to a great extent by molecular factors that either inhibit or stimulate growth, and as such, thorough characterization of these molecules will facilitate therapeutic interventions to promote brain rewiring.

1.4 Cell Transplantation Enhances Brain Plasticity After Stroke

Cell transplantation has shown much promise in experimental models of stroke with a diverse array of cell types including brain-, bone marrow-, blood-, and dental pulp-derived progenitors reported to enhance functional recovery after ischemic stroke (Chen et al. 2001a; Darsalia et al. 2011; Guzman et al. 2008a; Hicks et al. 2009; Kelly et al. 2004), reviewed in Bliss et al. (2007), and hemorrhagic stroke, reviewed in Andres et al. (2008a). Despite many preclinical studies showing that cell transplantation can improve recovery from stroke, the mechanisms mediating recovery are less understood. However, emerging evidence suggests that transplanted cells act to enhance endogenous repair mechanisms normally activated in the brain after stroke including brain plasticity (Andres et al. 2011a; Arvidsson et al. 2002; Bliss et al. 2010; Horie et al. 2011; Li et al. 2010; Liu et al. 2008; Ohtaki et al. 2008).

1.4.1 Transplanted Cells Promote Dendritic Plasticity

Fetal-derived neural progenitor cells can enhance dendritic branching in both the ischemic and contralateral hemispheres (Andres et al. 2011a). Compared with vehicle controls, human neural progenitor cell (hNPC)-transplanted rats showed significant enhancement of dendritic branching of Golgi-stained dendrites from layer V cortical pyramidal neurons at 2 weeks post transplantation in both hemispheres (Andres et al. 2011a). Dendritic changes in the contralesional hemisphere had abated at 4 weeks after transplantation; the effects in the ipsilesional hemisphere were sustained. This evidence suggests that dendritic proximity to hNPCs helps sustain branching. Increases in dendritic branching were most significant in basilar dendrites and middle-order branches; however, the significance of this for neuron function remains to be elucidated. The pattern of early dendritic changes in the contralesional cortex followed by a switch to more dominant changes in the ipsilesional cortex at later times is reminiscent of brain remapping results in patients and animals. These remapping studies show that stimulation of the injured limb early after stroke recruits the contralesional cortex and this switches back to the ipsilesional cortex at later time points (Benowitz and Carmichael 2010; Dancause 2006). It would thus be of interest to determine the significance of these dendritic changes to such remapping data.

1.4.2 Transplanted Cells Promote Axonal Rewiring After Stroke

Axonal sprouting occurs after stroke with new projections thought to target areas denervated by the stroke injury (Benowitz and Carmichael 2010). In rodent and primate models of ischemic cortical injury, such sprouting has been observed locally around the infarct area (Carmichael et al. 2001; Conner et al. 2005; Dancause 2006; Li et al. 2010), and interhemispheric axonal outgrowth from the intact cortex to the injured hemisphere has also been observed (Carmichael 2008). BDA axonal tracer studies from our lab and others showed that hNPCs (Andres et al. 2011a; Daadi et al. 2010) and human umbilical cord blood cells (Xiao et al. 2005) enhance interhemispheric cortical sprouting in corticocortical, corticostriatal, and corticothalamic pathways. In addition, hNPCs increased expression of the axonal growth cone protein GAP-43 in the corpus callosum and bilateral cortical hemispheres, with the largest change seen in the peri-infarct cortex (Andres et al. 2011a). However, GAP-43 is not purely a marker of regenerating axons as it is also expressed on nonneuronal cells such as astrocytes and oligodendrocytes (Carmichael 2008); the importance of this for regeneration is not understood. hNPC and mesenchymal stem cell (MSC) transplantation also enhanced stroke-induced remodeling of cortical spinal tract axons originating from the contralesional cortex (i.e., intact corticospinal tract) (Andres et al. 2011; Chen et al. 2002; Liu et al. 2008); such remodeling also included sprouting of the intact corticospinal tract into denervated regions of the spinal cord. Cell-induced changes in both corticospinal tract and transcallosal axonal sprouting statistically correlated with cell-enhanced functional recovery implying that cell-induced axonal plasticity is an important mechanism for stem- cell-induced recovery, although a direct causal link remains to be demonstrated.

Previous reports have demonstrated impairment of axonal transport following stroke (Wakita et al. 2002). Since axonal transport is fundamental to neuronal function and survival, we subsequently investigated the effect of transplanted hNPCs on anterograde axonal transport by measuring amyloid precursor protein (APP) accumulation in axons. We found that hNPCs significantly enhanced recovery of axonal transport following ischemia (Andres et al. 2011). Compared with vehicle controls, hNPC-transplanted rats had significantly fewer APP-positive axons at 3 weeks with further enhancement at 5 weeks. Using SMI312 immunostaining for axons, we also demonstrated more axons in the corpus callosum at 5 weeks in hNPC-treated animals. Moreover, APP accumulation in the corpus callosum negatively correlated with functional recovery in the whisker-paw and cylinder behavioral tests. These data suggest that transplanted hNPCs help improve axonal transport and enhance functional recovery.

This is a significant finding as axonal transport is fundamental to neuron function, not only for proper functioning and survival of existing axons but also for plasticity changes such as axonal sprouting and synaptogenesis. Therefore, hNPC-induced restoration of impaired axonal transport after stroke may not only enhance the function of existing fiber tracts but may also be a key upstream event of hNPC-induced structural plasticity.

1.5 How Do Stem Cells Modulate Brain Plasticity?

There are two mechanisms through which stem cells could modulate plasticity: (1) through secretion of factors that can modulate plasticity events, directly or indirectly, and (2) by integration into host circuits.

1.5.1 Transplanted Cell-Secreted Factors Modulate Dendritic and Axonal Plasticity

Neural progenitor cells and MSCs express many factors known to influence neurite plasticity (Kurozumi et al. 2005; Llado et al. 2004; Wright et al. 2003) including molecules such as neurotrophic factors like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (Himmelseher et al. 1997; Rosenstein et al. 2003), extracellular matrix molecules like the thrombospondins and secreted protein acidic and rich in cysteine (SPARC) (Au et al. 2007; Liauw et al. 2008; Osterhout et al. 1992), and factors important for neurite growth and guidance during development like Slit (Dancause 2006; Lin and Isacson 2006). Using immunodepletion experiments in a noncontact coculture assay of hNPCs with primary cortical neurons, we found that depletion of thrombospondins 1 and 2 or human VEGF significantly reduced hNPC-induced dendritic branching and length, while neutralization of Slit only affected total dendritic length (Andres et al. 2011). However, depletion of any of the above factors significantly reduced hNPC-mediated axonal outgrowth. In addition, using a microfluidic platform, we were able to show in vitro that neutralization of VEGF—not thrombospondins 1 and 2, SPARC, or Slit—inhibited hNPC-mediated effects on axonal transport. These data demonstrate that hNPC-secreted factors can modulate

neuronal plasticity at least in vitro. Whether these factors are also important for the in vivo effects of hNPCs and MSCs remains to be determined. However, using quantitative polymerase chain reaction analysis, we were able to identify the expression of VEGF and thrombospondins 1 and 2 in transplanted hNPCs in stroke brains 1 week post transplantation (Andres et al. 2011), and we also showed that hNPC-secreted VEGF is necessary for hNPC-induced recovery (Horie et al. 2011), thus suggesting a potential role for these molecules in vivo.

The exact effects of stem-cell-secreted factors on brain plasticity have yet to be elucidated. It is possible that they act through common pathways that affect axonal plasticity, possibly through downregulation of plasticity inhibitors or upregulation of activators (Benowitz and Carmichael 2010). For example, proteins that associate with CNS myelin, such as NogoA and myelin-associated glycoprotein (MAG), and scar tissue containing chondroitin and keratin sulfate proteoglycans at the ischemic injury site have been shown to inhibit axonal outgrowth (Benowitz and Carmichael 2010; Galtrey et al. 2007; Pizzorusso et al. 2002; Silver and Miller 2004). The effect of cell transplantation on such inhibitors is to date unknown. Stem cells may also act by activating the intrinsic growth potential of neurons. Expression of a number of growth-promoting molecules increases in the ischemic penumbra after stroke, and this gene expression pattern, not surprisingly, differs in the young versus aged brain (Carmichael et al. 2005; Li et al. 2010). In the young adult, many of the genes expressed in the sprouting neurons are linked to axonal sprouting or pathfinding during development (Li et al. 2010) including axonal guidance receptors such as neuropilin-1 (*Nrp1*) and L1 cell adhesion molecule (*L1cam*) and cytoskeletal modifying proteins such as stathmins (*Stmn3* and *Stmn4*). In contrast, aged sprouting neurons show a greater expression of immune-related genes, insulin-like growth factor-1 (*Igf1*) and bone morphogenetic proteins, and a distinct number of molecules that actually inhibit axonal growth such as the ephrin type-A receptor-4 (*Epha4*).

Transplanted cells could also enhance plasticity indirectly by modulating other processes that affect brain plasticity. For example, regrowth of blood vessels after stroke is important to support the survival and growth of neurons (and other brain cells) and undoubtedly will be necessary for brain plasticity. We and others have reported that hNPCs and hMSCs enhanced revascularization in the peri-infarct region after stroke (Chen et al. 2003; Hayashi et al. 2006; Horie et al. 2011; Onda et al. 2008). Moreover, by selectively immunodepleting hNPC-secreted VEGF using Avastin, an anti-human VEGF antibody that does not bind to rodent VEGF, we showed that VEGF secretion by the transplanted cells was essential for their enhancement of vascularization. Transplanted cells (hNPCs, MCSs, or human cord blood cells) can also modulate inflammation after stroke. They have been shown to not only downregulate inflammatory genes (Ohtaki et al. 2008) but also reduce the number of leukocytes in the brain (Horie et al. 2011; Lee et al. 2008; Vendrame et al. 2005). This immunomodulatory action of stem cells could influence brain plasticity not only by affecting neuronal survival but also by effects on synaptic plasticity, as many inflammatory cytokines (e.g., IL1, TNFα, IL6) can influence neural plasticity either in a beneficial or detrimental manner depending on their concentration (Yirmiya and Goshen 2011).

1.5.2 Integration into the Host Brain

The initial attraction of NPC cells for stroke therapy was their potential to become neurons and replace lost circuitry; however, evidence for this is limited. Transplanted NPCs in a rat model of global ischemia (Toda et al. 2001) and hNT neurons in a model of traumatic brain injury (Zhang et al. 2005) have been reported to express synaptic proteins. Electron microscopy studies revealed that human NPCs form synapses with host circuits after ischemia (Daadi et al. 2009; Horie et al. 2011; Ishibashi et al. 2004; Lee et al. 2008; Vendrame et al. 2005) and electrophysiological properties characteristic of functional neurons have also been shown for transplanted hNPCs (Buhnemann et al. 2006; Daadi et al. 2009). However, only very few synapses are seen, and recovery often occurs too early to be attributable to newly formed neuronal connections (Englund et al. 2002; Song et al. 2002), although such integration might be significant for recovery at later time points. Moreover, recovery is also reported with nonneuronal cells (e.g., MSCs) and when NPCs do not differentiate into mature neurons (Andres et al. 2011; Horie et al. 2011). Together, this implies that neuronal replacement is not necessary for cell-induced recovery, and the significance of the limited neuronal integration reported remains to be determined. NPCs also have the potential to become oligodendrocytes and astrocytes which could also integrate into the host brain and affect plasticity. Replacement of lost oligodendrocytes to remyelinate axons would be beneficial after stroke; remyelination by human NPCs was reported in spinal cord injury (Cummings et al. 2005); however, to date there are few reports of transplanted NPCs becoming oligodendrocytes in the ischemic brain (Daadi et al. 2008, 2009). Astrocytes play multiple roles in the brain including regulation of synapse formation and activity (Allen and Barres 2005), primarily through secretion of factors such as thrombospondins. Therefore, astrocytic integration could also be beneficial for stroke recovery.

1.6 Translational Implications of Stem Cell Therapy

Thrombolysis therapy using t-PA is the only means to improve functional recovery in the postischemic phase. This therapy, which aims to lyse the clot and restore blood flow, is limited in that it must be delivered within the first few hours after stroke. Many stroke patients are not eligible, however, because they arrive at hospitals well out of this time window. Cell transplantation therapies are not restricted to the same time limitations because they aim to enhance repair mechanisms. Most preclinical studies transplant within the first 3 days after stroke. Furthermore, studies have confirmed recovery within the subacute period (1 week post stroke) and chronic period (>3 weeks post stroke) (Andres et al. 2011; Borlongan et al. 1998; Chen et al. 2001b; Daadi et al. 2008; Horie et al. 2011; Pollock et al. 2006; Shen et al. 2007; Zhao et al. 2002). The timing of therapy should be considered as it may elicit differing clinical responses. To induce a neuroprotective response, acute delivery may be warranted whereas therapy during the first few weeks after stroke may promote repair mechanisms.

Proper patient selection will be crucial in determining the efficacy of stem cell therapeutics. Three factors should be considered in patient selection: patient demographics, lesion (location and size), and nature of stroke (hemorrhagic versus ischemic). Stroke typically affects the elderly population with significant comorbidities, such as hypertension, diabetes mellitus, and atherosclerosis. Aged rats have a less plastic gene expression profile after stroke (Li et al. 2010) and have higher astrocyte reactivity, increased macrophage recruitment, and delayed neuronal death after hemorrhagic stroke than younger rats (Wasserman et al. 2008). Therefore, further studies into the behavior of transplanted cells into young and mature brains need to be conducted.

Multiple means of cell delivery exist; the most common approaches are intracerebral, intravascular, and intracerebroventricular (Andres et al. 2008a; Bliss et al. 2007; Guzman et al. 2008b; Hicks and Jolkkonen 2009); however, the optimal means of delivery is still unknown. Intracerebral delivery may provide a greater number of transplanted cells than the other delivery routes. For larger stroke volumes, intravascular delivery may provide a wider distribution of cells to ischemic areas (Guzman et al. 2008b). Interestingly, many studies have shown significant recovery with a small number of cells (Guzman et al. 2008b; Hicks and Jolkkonen 2009; Li et al. 2002; Vendrame et al. 2004) or even no cells. In these cases, it is most likely that transplanted cells exert their effect through paracrine factors. Nevertheless, in the larger human brain, the question remains whether secreted factors must diffuse further or if they can act locally to alter surrounding circuits that alter remote remapping. Pending data from clinical trials should shed more light on these questions.

At a behavioral level, experiments in animals to favor the impaired limb by constraining the unimpaired limb have also resulted in increased axonal projections and synaptic densities and improved fine motor control (Maier et al. 2008). To design effective strategies for long-term recovery, therefore, paradigms to improve functional recovery after stroke should involve molecular aspects of synaptogenesis as well as behavior patterns through physical and occupational therapy.

Conclusions

Successful translation of stem cell therapy for cerebral ischemia requires not only answers to basic cell biology questions and knowledge of the molecular factors necessary for synaptogenesis and functional recovery but also identification of patient and donor cell factors as well as development of treatment protocols and outcome measurements (Abe et al. 2012). Patient factors include the appropriate age, stroke type, and patient comorbidities. Donor cell factors include identifying the optimal cell types, their safety profiles, and the safe and rapid protocol for ex vivo cell expansion. Treatment protocols include defining the timing for cell transplantation after the ischemic event, the best delivery route, and the most advantageous cell dose. Last, it is also important to choose the appropriate outcome measures, such as cell migration and integration tracking, brain function imaging, and behavioral functionality. Answering these questions successfully will require a great collaborative effort among scientists and clinicians. The data yielded thus far point to a promising future.

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Delivery Routes for Cell Therapy in Stroke

2

Alison E. Willing and Md Shahaduzzaman

2.1 Introduction

Stroke was not originally thought to be a good target for development of a neural transplantation therapy. Unlike Parkinson's disease in which a specific cell population is lost and therefore could conceivably be replaced, cell loss post stroke is not limited to a specific neuronal cell type or even neurons, making it more complicated to rebuild the neural circuitry. Even so, the first studies that used cell therapy for the treatment of stroke were performed over 20 years ago and published in 1988. In one study, fetal cortical neurons were transplanted directly into the cortex of adult rats that had undergone temporary middle cerebral artery occlusion (MCAO) (Mampalam et al. 1988). These grafts survived, developed appropriate neurotransmitter phenotype as demonstrated with nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) and acetylcholinesterase (AChE) expression, and had neurites that left the transplant and integrated into the host brain. The other study focused on a labeling strategy to identify the transplanted fetal neurons in the host after ischemia induced by 4-vessel occlusion (4VO) (Farber et al. 1988). Shortly, thereafter, another research group demonstrated that grafted fetal hippocampal neurons integrated into hippocampal CA1, receiving fiber ingrowth from septum that made synaptic contacts with the grafted neurons and projecting to posterior levels of host CA1 (Tonder et al. 1989). These early studies demonstrated proof of principle that fetal neurons could survive and engraft in infarcted brain. Subsequent studies examined the ability to rebuild neural circuits and reduce functional deficits.

In these earlier days of cell therapy, it was inconceivable that cells could be transplanted outside the central nervous system (CNS) and have a therapeutic effect. Therefore, all the early studies employed either direct implantation into the injured

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brain (parenchymal) or into the nearby ventricular system (intracerebroventricular or i.c.v.). In more recent years, observations that bone marrow-derived cells could enter the brain and express microglial or astrocytic antigens after intravenous (i.v.) administration (Eglitis and Mezey 1997) led researchers to ask if other routes of cell delivery could be efficacious. The first report of i.v. delivery examined cell efficacy in a rodent model of traumatic brain injury (Lu et al. 2001), followed shortly thereafter by a publication from the same research group demonstrating that intra-arterial (i.a.) administration of bone marrow stromal cells was effective at inducing functional recovery in a MCAO model of stroke (Li et al. 2001a). Since that time, the literature on cell therapies using these nontraditional routes of delivery has greatly expanded. In fact, when the route of administration is compared over time, there is a shift in the route of administration that is predominantly used, based on the cell types that are studied (Table 2.1). In this chapter, we will not provide an exhaustive review of the field, but we will provide a short overview of the cell therapy literature focusing on the benefits and risks of these different routes of administration, discussing intraparenchymal, vascular, and ventricular routes of administration.

2.2 The Intraparenchymal Route of Cell Delivery

All the early neural transplantation studies for stroke used either the intraparenchymal or intraventricular route of delivery of fetal neurons. The goal of these studies was predominantly to determine the feasibility of replacing lost neurons and rebuilding neural circuits. The site of transplantation depended on the stroke model employed. For example, in the four-vessel occlusion model, neurons are lost in CA1. Transplants of fetal hippocampal neurons from embryonic day 17–19 (E17–E19) rats survived well for extended periods of time (>100 days post transplant) and expressed appropriate region-specific receptors (Aoki et al. 1993). Further, the cells significantly improved performance on a spatial memory task (Netto et al. 1993). Transplantation of neural stem cells (NSCs) in this region also produces recovery if sufficient cells survive and express neuronal proteins (Toda et al. 2001). Using the similar bilateral occlusion model, investigators have transplanted NSCs into cortex, hippocampus, or striatum, depending on the experimental question to be addressed (Shichinohe et al. 2010; Ohtaki et al. 2008; Nodari et al. 2010).

The focus of the studies using this route of delivery depends on the cells being studied. In those studies that have transplanted primary fetal neurons, the experimental questions concerned whether the grafts survived, for how long, and if survival could be modified (Koshinaga et al. 1995). They also asked whether the fetal neurons could mature into an adult phenotype that was appropriate for the region they were transplanted into (Mampalam et al. 1988; Nishino et al. 1993a). Did these cells express neurotransmitters and have neuritic processes from graft and host developing synaptic contacts (Mampalam et al. 1988; Grabowski et al. 1992a, b; Onizuka et al. 1996; Aihara et al. 1994; Belichenko et al. 2001)? Most importantly, questions of the ability of the cells to decrease infarct volume (Johnston et al. 2001) and improve motor, cognitive, or somatosensory function were also

Table 2.1 Routes of cell administration for treatment of experimental stroke: analysis over time[a]

Year	Cell type	# of studies	Route				Primary outcomes[b]
			Parenchymal	Vascular	Ventricular	Other	
1991[c]	Fetal neurons	3	3				2, 3, 5, 7, 12
	NSCs						
	Bone marrow						
	Umbilical cord						
	Other	1	1				2, 6, 12
2001	Fetal neurons	2	2				1, 3, 5, 12
	NSCs	2	2				1, 2, 14, 15
	Bone marrow	5	2	3			2, 3, 4, 5, 6, 8, 9, 10
	Umbilical cord	1		1			2, 3, 5, 6, 9
	Other	4	4				1, 2, 3, 7, 12
2011	Fetal neurons	1	1				3, 5, 12
	NSCs	13	7	3	2	1	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12
	Bone marrow	23	5	18			1, 2, 3, 5, 6,7,8, 9, 10, 11, 12, 13, 14, 15, 16
	Umbilical cord	5	1	3		1	1, 2, 3, 7, 9, 10, 11, 12, 13, 16
	Other	2	1	1			1, 2, 3, 5, 6, 7

[a]The analysis was performed using the OVID and PUBMED literature database programs. The search strategy was as follows: (cerebral ischemia or cerebral hemorrhage or cerebrovascular accident or stroke) AND (cell therapy or cell transplantation or fetal tissue transplantation or fetal neurons or bone marrow or umbilical cord or mesenchymal stem cells, stem cells, endothelial progenitor cells)

[b]Primary outcome variables:
(1) infarct size, (2) functional outcome, (3) cell survival, (4) proliferation, (5) differentiation, (6) migration, (7) trophic support, (8) anti-inflammatory, (9) cell dose, (10) induction of neurogenesis, (11) induction of angiogenesis, (12) dendritic/synaptic plasticity, (13) cortical reorganization, (14) safety, (15) feasibility, (16) timing

[c]Includes years 1988–1991

addressed (Zeng et al. 1999; Nishino and Borlongan 2000; Nishino et al. 1993b; Borlongan et al. 1998a, b). Later studies that tested NSCs as a treatment for stroke not only examined these same issues but were also concerned with the ability of the stem cells to proliferate (Darsalia et al. 2007), differentiate into appropriate neuronal types (Darsalia et al. 2007), and migrate to the site of injury (Darsalia et al. 2007; Hoehn et al. 2002; Lee et al. 2010a). The studies that have used non-neural cells as potential treatments have addressed issues of the ability of the cells to transdifferentiate, becoming neurons, astrocytes, or oligodendrocytes (Chen et al. 2001a, b). Alternative mechanisms of recovery such as trophic support

(Chang et al. 2002; Ferrer et al. 2001; Lin et al. 2011), anti-inflammation (Shen et al. 2010), and induced neurogenesis (Li et al. 2001b) or angiogenesis (Lee et al. 2010a) were also explored.

2.2.1 Intraparenchymal Routes of Delivery in Clinical Studies

The first cell therapy study for stroke patients was conducted in the late 1990s and the first paper published in 2000. The cells chosen were the LBS neurons, derived from a human teratocarcinoma, that had been shown in earlier studies to differentiate into neuron-like cells (Andrews 1984; Lee and Andrews 1986; Pleasure et al. 1992; Thompson et al. 1984; Trojanowski et al. 1993). In animal studies, these cells improved outcome after direct transplantation into the striatum of rats subjected to MCAO (Borlongan et al. 1998a, b). In this study, LBS neurons were injected into 12 patients with fixed deficits after lacunar stroke. European Stroke Scale score improved and PET scans performed 6 months post transplant showed increased metabolic activity at the implant site. Upon post-mortem examination from the first deceased patient, the cells did survive and express neuronal antigens; no tumor growth was detected (Nelson et al. 2002). The second phase II study was published in 2005 (Kondziolka et al. 2005). In this study, 5 or 10 million LBS neurons derived were transplanted into 25 sites in the brains of nine patients with fixed motor deficits after subcortical ischemic stroke and 9 patients with fixed motor deficits after hemorrhagic stroke. There were some improvements in motor function observed, but the primary endpoint did not change. Later analysis of cognitive function showed that some of the patients had marked improvement (Stilley et al. 2004). There were no adverse effects observed (Kondziolka et al. 2004). The study demonstrated safety and feasibility, but not efficacy.

2.3 The Vascular Delivery Route

The vascular delivery route became more common with the demonstration that bone marrow stromal cells could induce functional recovery even when they were delivered i.v. (Lu et al. 2001). As demonstrated in Table 2.1, there are now an abundance of studies that show that both i.v. and i.a. delivery are efficacious in animal models of stroke.

2.3.1 Intravenous

There have been a number of animal studies that have examined the ability of cells (bone marrow cells, umbilical cord cells, neural stem cells, etc.) delivered i.v. to reduce infarct size or, more importantly, to induce functional (motor and/or cognitive) recovery. The specific vein that the cells are injected into may vary, but the most common are the tail vein and jugular vein, although femoral and penile are

also common. In 2001, the first i.v. bone marrow stromal cell transplants (Chen et al. 2001c) and human umbilical cord blood cells (Chen et al. 2001b) for stroke studies were published. The goal of both studies was to demonstrate that the cells could improve motor and cognitive function after stroke and set the basic parameters for the timing of transplantation. In both studies, the cells produced the greatest recovery when administered at 24 h post stroke. When comparing across studies, the cord blood seemed to be marginally better at inducing recovery on the Rotarod test (~85 % compared to 75 % of baseline). There was no effect of cell delivery on infarct size and only about 10 % of the transplanted cells were present in the infarcted hemisphere in either study. In later studies that looked at the biodistribution of those cells, when MSCs, NSCs, multipotent adult progenitor cells (MAPCs), or bone marrow mononuclear cells were injected i.v. in a normal rat, less than 1 % of the MSCs, NSCs, or MAPCs were observed in arterial circulation, with most of these cells being found in lung, kidney, spleen, and liver (Fischer et al. 2009). The exception was the bone marrow mononuclear cells which are smaller than the other cells. Fully 5 % of these cells reached arterial circulation, although the majority of the cells were found in the kidney, spleen, and liver.

Even while survival of human umbilical cord blood cells in the brain was minimal, these cells consistently improved outcome after MCAO. In our subsequent studies, we determined that i.v. delivery was better than intraparenchymal delivery in the injured striatum (Willing et al. 2003), we optimized the number of cells necessary to maximize behavioral recovery and minimize infarct size (Vendrame et al. 2004) and delineated the ideal timing of cell delivery (Newcomb et al. 2006). While we and others showed that the cells could directly interact with all neural cells (Dasari et al. 2008; Hall et al. 2009a; Jiang et al. 2010, 2011) and they could migrate toward extracts of infarcted brain (Chen et al. 2001b; Jiang et al. 2008; Newman et al. 2005), the cells did not have to enter the brain to induce recovery (Borlongan et al. 2004; Nystedt et al. 2006; Makinen et al. 2006). The beneficial effects of i.v. HUCB delivery included local (Vendrame et al. 2005; Leonardo et al. 2010) and systemic (Vendrame et al. 2006; Hall et al. 2009b) anti-inflammatory properties as well as induction of neurogenesis and angiogenesis (Taguchi et al. 2004). In the bone marrow literature, similar findings were observed (Li et al. 2001b; Barbosa de Fonseca et al. 2010; Chen et al. 2002, 2003; Le et al. 2010; Shen et al. 2006).

Perhaps a more surprising application of the i.v. route of delivery has been for delivery of NSCs. One of the advantages of NSC treatments was assumed to be their ability to differentiate into neurons, astrocytes, and oligodendrocytes in order to rebuild the local neural structure. But in recent years, it has become clear that the environment in the injured adult brain is not optimal for this and recovery after NSC treatment is actually occurring through growth factor-mediated or anti-inflammatory processes similar to those observed with bone marrow or HUCB cells (Sun et al. 2010; Lee et al. 2008). For example, in the collagenase model of intracerebral hemorrhage, i.v. administration of human NSC decreased both brain and spleen cytokine expression and splenectomy reversed this effect (Lee et al. 2008). NSCs respond to many of the same chemotactic cues as marrow- or cord blood-derived cells, so it

was not too far-fetched to believe that they could also migrate to the site of injury when administered by a vascular route. Minnerup and associates recently reported that neural progenitor cells administered i.v. improved performance on the adhesive removal test of rats that had previously undergone photothrombotic stroke (Minnerup et al. 2011). While few of the LacZ-labeled cells were identified in the injured cortex, those that were expressed doublecortin suggesting they were going to become neurons. Even more interesting, these cells did not induce endogenous neurogenesis, but they did enhance dendritic outgrowth leading the authors to postulate that this was the mechanism underlying behavioral recovery. When embryonic stem cells were injected i.v. and migration examined with SPECT imaging, they also did not migrate to brain (Lappalainen et al. 2008).

2.3.2 Intra-arterial

It has been suggested that the i.a. route of delivery is superior to the i.v. route because the cells would be directly delivered to the brain where they could act to decrease infarct size and increase functional recovery. The first report of i.a. cell administration involved the injection of BrdU-labeled BMSCs through the internal carotid artery 24 h after transient MCAO (Li et al. 2001a). Compared to vehicle-treated controls, cell-injected animals scored significantly better on the neurological severity score and the adhesive removal test, but there were no significant differences in infarct size between groups. Approximately 21 % of the delivered cells were observed in the infarcted hemisphere, but no data were reported on distribution of the rest of the transplanted cells. Later studies using noninvasive imaging techniques found that by 24 h post injection, 95 % of the injected cells were found in the spleen (Keimpema et al. 2009). In follow-up studies, i.a. transplantation increased angiogenesis and proliferation of NG2-positive oligodendrocyte progenitors (Shen et al. 2006).

As with the i.v. route of delivery, NSCs or neural progenitor cells have also been delivered by the i.a. route. There are a number of studies that have reported injecting neural stem or progenitor cells via this route. The first study was a side-by-side comparison of intraparenchymal, i.v., and i.a. transplantation of human ES-derived neural cells and rat hippocampal cells. After i.v. delivery, the cells were found mainly in the liver but also in the spleen and kidney using SPECT imaging; no cells were found in the brain (Lappalainen et al. 2008). With i.a. delivery of the human ES-derived cells, the cells were also found in the brain, but not to the same extent as was found with direct implantation of rat hippocampal neurons. In another study, red fluorescent protein-labeled cells were tracked using bioluminescence imaging (Pendharkar et al. 2010). After i.a. delivery, the fluorescent signal was observed in the head region; this signal was still visible 7 days later. With the i.v. route, the fluorescent signal was only visible in the torso and was not evident 7 days post injection. These results were verified with SPIO labeling of neural stem cells and histology. Immediately after i.a. injection, the cells were present in the vasculature, but by 2 weeks post injection they were observed in the parenchyma. In the third study, the i.a., i.v., and intracisternal routes were compared (Li et al. 2010). Within 4 h, magnetic-labeled NPCs were observed in the infarcted hemisphere. With intracisternal

and i.v. delivery, the cells only appeared in the infarcted hemisphere 2–3 days later and there were significantly fewer of these cells. These results are generally consistent with the observations of the previous study. What is more interesting is that mortality in the three groups was significantly different. Forty-one percent of animals in the i.a. group died compared to 8 % in the i.v. group, which was similar to MCAO only (10 %). So while these studies both suggest that i.a. is the preferred route of delivery when it is necessary to get cells into the brain, the high mortality would suggest that caution should be used in employing this route.

2.3.3 Vascular Routes of Delivery in Clinical Studies

Both i.a. and i.v. routes have been used in clinical trial of bone marrow-derived cells. In the first study, just as reported in the animal studies, most of the cells were found in liver, lung, spleen, kidney, and bladder after i.a. delivery (Battistella et al. 2011). Only in two patients were the cells observed in the brain, but even at 6-month follow-up there were no adverse events. Another study that examined i.v. administration of bone marrow mononuclear cells also found no adverse effects that were attributable to the cell infusion (Savitz et al. 2011). Patients exhibited functional improvements on multiple neurologic scales out to 6 months. Intravenous administration of MSCs has also been performed (Bang et al. 2005). In this study, five patients with severe neurologic deficits after a stroke in the MCA territory received a total of 10^8 autologous MSCs over two injections. Imaging was performed to determine infarct volume and National Institutes of Health Stroke Scale (NIHSS), Barthel Index, and modified Rankin Scale for functional recovery. There were no significant differences at study enrolment between these patients and the control group of untransplanted patients ($n=25$). Lesion volume did not change over the ensuing year, although ventricular dilation was significantly more prominent in the control patients. There were significantly improved scores on the Barthel Index and a tendency toward improvement on the modified Rankin Scale. This research group expanded the initial study to examine survival and long-term outcomes of stroke patients with ($n=16$) or without ($n=36$) MSC transplantation after 5-year follow-up (Lee et al. 2010b). Mortality of the transplanted patients was 25 % compared to 58.3 % of the control group. There were significant improvements in the modified Rankin Scale scores of the treated group, and no difference between groups in comorbidities (such as seizures) and no side effects observed. Taken together, these data suggest that i.v. administration of MSC is safe and efficacious.

2.4 Administration into the Ventricular System

There are a few studies that have examined the ability of cell transplants in the ventricular system (i.c.v., intracisternal, or intrathecal) to migrate to the infarcted hemisphere, integrate into the local brain circuitry, and induce anatomical and functional repair in a stroke model. One of the issues, especially when there are early progenitors or stem cells within the cell preparation, is the overgrowth of the ventricles.

Folkerth and Durso (1996) published a case report in which a Parkinson's patient that had received i.c.v. transplants from a fetus 5–6 weeks of age died suddenly 23 months after transplantation. Upon autopsy, the grafts which filled the left lateral ventricle and the fourth ventricle were composed of mesenchymal and ectodermal cells, but not neurons.

2.4.1 Intracerebroventricular Route

In 1999, Kopen and associates demonstrated that bone marrow stromal cells became integrated into forebrain and cerebellum by 12 days after they were injected into the lateral ventricles of neonatal rats (Kopen et al. 1999), demonstrating that the cells were capable of migrating into the brain. While this study provided evidence that cells administered by the i.c.v. route could enter the brain, there have been few studies that have followed suit. One exception was a study that used transplantation of microglia 1 h after MCAO induction to examine the role of microglia in neuroprotective repair after injury (Kitamura et al. 2004). Those animals that were injected with microglia had significantly more neurons surviving in lesioned cortex than did vehicle-treated controls.

2.4.2 Intracisternal

When NSCs derived from subventricular zone were injected, MRI was used to track ferromagnetic-labeled NSCs from young adult rat when they were injected into the cisterna magna 48 h after MCAO (Zhang et al. 2003). Fully 85 % of the MRI signal was observed in the ischemic striatum; almost 6,000 labeled cells of the 100,000 transplanted were present by 35 days post transplant. Functional recovery on the foot fault and adhesive removal tests was observed in transplanted animals. The 6 % survival rate of the transplanted tissue is similar to that observed in other studies of neuronal transplantation.

2.4.3 Intrathecal

There have been few studies that have examined the efficacy of administering cells intrathecally. In a recent article, Seyed Jafari and colleagues (2011) examined whether adult PKH-26-labeled neural stem cells (NSCs) administered by lumbar puncture could migrate to the infarcted brain and develop into neurons and astrocytes. They found labeled cells expressing S100 and β-tubulin floating in the ventricles and attached to the ventricular wall 1 month after transplantation. Cells were not observed around the infarct, and while performance improved significantly on the Rotarod test of motor coordination, performance was still considerably impaired. Further, these authors provided no indication of how many cells were found in the brain ventricles or where else they were found. This will be critical to determine based on a recent report of an ataxia-telangiectasia patient that received NSC transplants both intracerebellar and intrathecally (Amariglio et al. 2009). Within 6 months

of treatment, the patient had developed both a brainstem tumor and a tumor at level L3–4 of the spinal cord attached to the cauda equina nerve roots; there were satellite tumors around both of the larger masses. Molecular analysis of the cauda equina tumor suggested it was derived from the NSC donor.

2.5 Other Routes of Delivery: Intraperitoneal

This route of delivery has only been applied in the stroke field in rat models of neonatal hypoxia-ischemia. The first study describing this approach injected MSC i.p. 2 h after birth and then determined location and phenotype of the cells 14 days postnatally. More cells were found in the ischemic hemisphere than on the contralateral side and few expressed neural proteins (Guan et al. 2004). No behavioral measures were employed.

More recently, two studies have expanded on this early work. In the first study, the Rice-Vannucci model of neonatal hypoxia-ischemia was used to determine the ability of human cord blood-derived mononuclear cells to repair the damaged neonatal brain (Pimentel-Coelho et al. 2010). The cells were injected 3 h after neonatal hypoxia-ischemia and a neurologic testing regimen including the cliff aversion reflex, the negative geotaxis reflex, and gait, was examined. Cord blood cells improved reflex behavior, but not gait and decreased cell death.

In the second study, Geißler et al. (2011) showed that HUCB cells were able to migrate into the brain after hypoxia-ischemia at 7 days as determined with immunohistochemistry for HLA-DR. Again no indication of how many cells survived in the brain or distribution to other tissues was given. The cells were able to induce more long-term recovery on the forelimb asymmetry test that appeared to be the result of a reorganization of cortical maps controlling sensorimotor function as determined electrophysiologically.

The choice of an i.p. delivery route is based more on ease of delivery in the neonate than any other consideration. It is not clear that this route would be efficacious in an adult. Barrier function in the neonate may not be as immutable yet as in the adult. Growth and trophic factor expression in the neonate may be more amenable to long-range migration and integration of the transplanted cells into the injured brain. These are cells that are usually found in blood, bone marrow, or lymphoid organs. They are usually extravasated into tissue from blood in response to injury or disease. They do not usually get taken up into the blood stream directly by "intravasation." The more likely route of uptake is through lymphatic drainage of the peritoneal cavity. Further, neither research group offers an explanation of how the cells may affect functional recovery with this delivery route. These questions remain to be answered.

2.6 What Is the Best Route for Cell Delivery?

Very few studies have directly compared routes of administration. In the earliest such study, we directly compared intraparenchymal into striatum and i.v. delivery of human cord blood-derived mononuclear cells after permanent MCAO (Willing

et al. 2003). Long-term functional recovery was better with i.v. delivery; at one month post MCAO, both groups demonstrated good recovery on a battery of behavioral tests, but at two months that recovery was only maintained by the group that received i.v. cells. In fact, performance of the group receiving cells into the striatum was even worse than the MCAO-only group, demonstrating that testing at longer post-stroke survival times is essential. As discussed earlier in this chapter, comparisons among the vascular routes have also been performed, demonstrating that cells do appear in the brain after i.a. delivery, but not after i.v. or intracisternal delivery (Li et al. 2010). There are no consistent studies that look at functional recovery and infarct size across all routes of delivery. When choosing the delivery method for a study, the best approach is to first ask what the underlying mechanism of repair is. For example, if we believe that human umbilical cord blood cells reduce infarct size and improve functional outcomes by altering the systemic inflammatory milieu, then it does not make sense to implant them directly into the brain. If, on the other hand, we believe that the major benefit of a NSC therapy is through the cells ability to differentiate into neurons, astrocytes, and oligodendrocytes, then it is more appropriate to deliver them directly into the brain or i.a. than to inject them i.v. Other benefits often attributed to the i.v. route, such as lower cost, relative safety, and ease of delivery, are of minor consideration next to this. Safety risks such as demonstrated increased risk of mortality with the i.a. route or tumorigenesis must also be considered.

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Different Sources of Stem Cells for Transplantation Therapy in Stroke

3

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3.1 Introduction

Historically, bone marrow has been the primary source of adult-derived stem cells; yet, as research has progressed, alternative sources of stem cells have been discovered. These various tissue-derived adult stem cells may be utilized as possible clinical therapies for the treatment of stroke. In this chapter, we will provide insight of the different tissues used to harvest stem cells, along with their limitations and advantages. An overview of the stem cells currently under investigation, from a neurorestorative standpoint, has previously been published (Huang et al. 2010).

3.2 Autologous Versus Allogenic

An important factor to consider in choosing stem cells for therapy is the use of autologous or allogenic cells. Autologous stem cell treatment involves procuring the cells from the same individual in which the cells will be used, compared to receiving cells from a donor, as is the case in allogenic stem cell transplantation. The condition of receiving stem cells from a donor may predispose an individual to immunogenic complications, including rejection. Furthermore, limitations between sources also include cell yield, ease of harvesting, proliferation capacity, and cell immunogenicity.

Limitations with cell yield and proliferation capacity, related to stroke, are evident in the research of optimal time for delivery with umbilical cord blood. Ideally, the

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delivery time for these stem cells is 48 h post stroke (Newcomb et al. 2006), which is still a short window of opportunity that poses as a challenge especially for generating an ample supply of stem cells from freshly harvested autologous tissue sources. As will be discussed with neural stem cells, ease of harvesting has a great influence over the practicality of therapeutic potential. Some of the harvesting techniques may require invasive procedures to acquire the stem cells.

Another barrier of stem cell treatment for stroke involves immune reactions elicited by the transplanted stem cells and the host tissue, as well as the immunosuppressants if used as an adjunct therapy for transplantation. A recent study suggests that the use of cyclosporin A promotes recovery from cortical injury following a stroke due to promotion of endogenous neural stem cell activity and migration (Erlandsson et al. 2011). Stroke studies in immunocompromised animals have demonstrated elevated endogenous neurogenesis via a CD4+ T cell, but not a CD25+ T cell-dependent mechanism (Saino et al. 2010). Although stem cells may produce an immunogenic response, it is evident that the more naïve or less lineage specific a cell is, the less likely it is to invoke an immune response. For instance, umbilical cord blood transplantation is less likely to require immunosuppression due to its immunological immaturity. As a result, human leukocyte antigen (HLA) matching is less strict preceding transplantation, while cell viability remains high compared to the requirements for bone marrow transplants (Willing et al. 2007). Some cells may be more immuno-inductive than bone marrow. Placental mesenchymal stromal cells (MSCs) are considered less immune immature, and although this tissue is composed of a heterogenous population of adult and neonatal cells (Fazekasova et al. 2011), chorionic plate-derived cells may illicit a greater immune response than other sources (Lee et al. 2012).

Today, there are many umbilical cord and tissue banks that can serve as storage for autologous or allogenic stem cells, and as the utilization of these stem cells continues to be developed, these repositories will serve to increase their accessibility.

3.3 Adult Stem Cells

A primary challenge with alternative sources for stem cells begins with determining the constitution of a cell population and how to then purify it. Because of both the diverse nature of stroke and stem cell mode of action, consideration is being taken as to the use of specific stem cell derivatives for treatment of particular stroke conditions.

3.4 Bone Marrow-Derived Stem Cells

The bone marrow hosts a diversity of cells that can be used as a mixture or purified to a precise cell type of interest. Emergent research within the last decade suggests the feasibility of bone marrow-derived stem cells for stroke therapy. Data demonstrates that bone marrow-derived stem cells can mobilize from the bone marrow

(BM) to the peripheral blood (PB), which can then eventually enter the central nervous system to influence neuronal injury (Borlongan et al. 2011). Bone marrow-derived cells include hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), endothelial progenitor cells (EPCs), and very small embryonic-like cells (VSELs) (Herzog et al. 2003). In this section, we will outline the use of these bone marrow-derived stem cell lines in the potential therapy for stroke.

Hematopoietic stem cell (HSC) mobilization can be induced by CNS cytokine production under stressful conditions, such as stroke (Lapidot et al. 2005; Lapidot and Kollet 2010; Nervi et al. 2006; Papayannopoulou and Scadden 2008). Neurotransmitters, notably catecholamines, can induce HSC mobilization through a nerve ending paracrine signal directly into bone marrow or through sympathetic release into blood circulation (Kalinkovich et al. 2009). Treatment protocols, such as granulocyte-colony-stimulating factor (G-CSF), apply this cytokine-mediated recruitment (Nervi et al. 2006; Papayannopoulou and Scadden 2008). Human clinical data of acute stroke shows an abundant mobilization of peripheral blood immature hematopoietic CD34+ cells, colony-forming cells, and LTC-IC (Hennemann et al. 2008), with the magnitude of mobilization correlating to recovery of function (Dunac et al. 2007). Beyond treatment for just stroke, the use of BM-derived HSC is heavily considered for disease conditions including cardiovascular, bone, and cartilage.

Mesenchymal stem cells (MSCs) are found in nearly all tissues of the body. Those residing outside of the bone marrow will be elaborated in a subsequent section, but in this section, we will discuss BM-derived MSCs for the treatment of stroke. The use of BM-derived MSCs elicits functional recovery of neurological deficits in the treatment of cerebral ischemia in stroke models (Bang et al. 2005; Chopp and Li 2002; Rempe and Kent 2002; Song et al. 2004). Further reports indicate that transplantation of MSCs may even produce therapeutic benefits without adverse side effects (Bang et al. 2005). A limitation of MSC therapy is the mode of delivery. Very low graft survival rates have been observed with transplantation by means of intravenous, intracarotid, or intracerebral delivery methods (Shen et al. 2007). Consequently, the benefits of MSC transplantation may be imparted by the introduction of neurotrophic factors that activates endogenous brain tissue, factors including hepatocyte growth factor (HGF) (Chopp and Li 2002; Chen et al. 2002), vascular endothelial growth factor (VEGF) (Chen et al. 2003), nerve growth factor (NGF) (Li et al. 2001), brain-derived neurotrophic factor (BDNF) (Li et al. 2001), basic fibroblast growth factor (bFGF, FGF-2) (Chen et al. 2003), and insulin-like growth factor-1 (IGF-1) (Zhang et al. 2004). In addition to secreting factors, the presence of MSCs may promote endogenous induction and migration of primary stem cells from their usual locations (SVZ and SGZ) to the location of injury while also reducing apoptosis in the penumbral zone of the lesion (Chen et al. 2003; Li et al. 2001).

Stroke can often be a consequence of disruption in vascular integrity. The permeability of the blood-brain barrier and stroke recovery can be related to endothelial modulation. Endothelial progenitor cells (EPCs) are precursors for the mature endothelium that lines the vascular system, a role that has long been determined.

In an early study, transplanted EPCs were found in newly vascularized endothelium of ischemic brain injury (Asahara et al. 1997). More recent research indicates that circulating BM-derived EPCs are signaled to sites for neovascularization, where they will differentiate into endothelial cells (Masuda and Asahara 2003). The research of EPCs and stroke-related vascularization is still narrow, but it is evident that they could play an integral role in the prevention of stroke and the treatment after an injury.

Very small embryonic stem cells reside in adult tissues and are mobilized into the peripheral blood following a stroke event (Ratajczak et al. 2010). The current hypothesis is that VSELs are epiblast-derived PSCs that are deposited early on during embryonic development (Borlongan et al. 2011), serving as a reserve within the tissue that can be utilized for rejuvenation. The brain is one such location that includes a large number of cells displaying the VSEL phenotype (Kucia et al. 2005; Zuba-Surma et al. 2008). A major possibility for stroke therapy is the ability for microglia VSELs to differentiate into neurons, oligodendrocytes, and macroglia to regenerate damaged CNS (Borlongan et al. 2011). However, some limitations currently exist when considering the use of VSELs, one restraint being the low number of VSELs that can be harvested. This would require the possibility of proliferation before transplantation (Borlongan et al. 2011). Another restriction is the decrease in number of VSELs with age, making harvesting an adequate number even more difficult in older individuals (Ratajczak et al. 2011).

3.5 Neural Stem Cells

For the treatment of stroke injury, the use of neural stem cells (NSCs) seems like an obvious solution. Endogenous stem cells are located in the subgranular zone (SGZ) of the dentate gyrus, the subventricular zone (SVZ), and the subependymal zone (SEZ) of the spinal cord. As may be expected, the cellular activity is upregulated in these zones following a stroke-like injury; yet, this activity does not provide cell replacement or full functional repair (Nakayama et al. 2010). Earlier studies support this notion by examining the migration of new cells.

There is a noted reduction in migration of new cells in the striatum after an insult, and upon migration, these cells did not differentiate into neurons. One proposition suggests that the area of injury produces a microenvironment that impedes cell replacement (Nygren et al. 2006; Deierborg et al. 2009). Another consideration is that maturation of the neural stem cells may be slower after ischemia (Tonchev et al. 2005; Taupin 2006). Stem cells from the SVZ may be redirected from their normal route through the rostral stream into a redefined direction to reach ischemic regions of the brain (Thored et al. 2007; Kojima et al. 2010). It appears as if chemokine signals such as stromal-derived factor-1 (SDF-1), vascular endothelial growth factor (VEGF), and angiopoietin are released from ischemic tissue, influencing the course of the SVZ NSCs toward a path along blood vessels to reach the infracted area (Barkho et al. 2008; Liu et al. 2009; Zhang et al. 2009; Carbajal et al. 2010).

While endogenous cell transport may not reach the lesion, current literature is evident that exogenous stem cell transplantation may elicit endogenous stem cell

production at the site of injury (Bachstetter et al. 2008; Park et al. 2010; van Velthoven et al. 2010; Jin et al. 2011). Although dendritic plasticity is increased, studies also indicate that endogenous neurogenesis may be impeded due to microglia activation post stroke (Minnerup et al. 2011). Despite this finding, microglia might perform differently in the presence of neural stem cells than they do in their absence. Rat models have demonstrated, in ex vivo cultures, a promotion in neurogenesis by the presence of microglia when treated with SVZ NSCs after an experimentally induced stroke (Deierborg et al. 2010).

The potential for neural stem cells to promote a microenvironment favorable for endogenous stem cell proliferation still has its limitations. A primary limitation is the procurement of these cells. An autologous treatment would require invasive surgery prior to therapy and allogenic grafts would likely require a fetal source or derivation from an alternative cell type. Another possibility would be harvesting the cells during other surgical procedure (Chaichana et al. 2009), but this may not be very advantageous. An even greater concern is the potential of stem cells to be tumorigenic.

Somewhat contrary of immunogenicity, the less differentiated the cell, the greater the potential for the cell line to generate aberrant proliferation. Thus, adult stem cells are less likely than embryonic stem cells to encourage tumorigenesis. When utilizing stem cells, it is essential to ensure the use of a purified cell population in transplantation (Jandial and Snyder 2009; Amariglio and Rechavi 2010). A prior case outlined this requirement when a child with ataxia-telangiectasia was transplanted with a heterogenous mixture of fetally derived neural stem cells and was diagnosed with a glioneuronal neoplasm 4 years later (Amariglio et al. 2009).

The reduced tumorigenic potential of adult-derived stem cells may be, in part, due to their reduction in proliferation capacity. Unfortunately, this presents a problem when attempting to achieve a sufficient number of stem cells for transplantation. To circumnavigate this problem, researchers have developed methods such as long-term culturing, immortalization, insertion of oncogenes, or even deriving neural stem cells from other tissues or from pluripotent stem cells. Each of these methods creates limitations as well. Long-term culturing has the risk of spontaneous conversion to a nonneural cell type such as a tumor precursor cell (Wu et al. 2011). In spite of the teratocarcinoma-derived hNT neuron cell lines advancing into a phase II clinical trial in stroke patients (Newman et al. 2005), no significant improvements were observed (Kondziolka et al. 2005). The insertion of the oncogene may still have a promising future. ReNeuron Ltd, a stem cell therapeutics company based in England, is using a c-Myc regulator gene and mutated estrogen receptor transgene to generate an immortalized neural cell line (Pollock et al. 2006). Currently, this protocol is undergoing clinical trials for stroke in the United Kingdom (Mack 2011).

3.6 Mesenchymal Stromal Cells

Mesenchymal stromal cells may be the most commonly studied stem cell studied from extraembryonic tissue. In contrast to the ectoderm-derived nervous system that is host to neural stem cells, which reside and migrate within the nervous system,

mesoderm-derived mesenchymal stromal cells (MSCs) are ubiquitously found in nearly all tissues of the body and may migrate to the nervous system in response to injury, although it is still contended whether these cells can differentiate into fully functional neurons (Burns et al. 2006; Breunig et al. 2007; White 2011). MSCs can be obtained from almost all tissues of the body, including bone marrow, placenta, teeth, and adipose tissue. This makes mesenchymal stem cells a favorable line for autologous transplantation. However, not all MSCs are created equal.

Evidence reports that mesenchymal stem cells from different locations may impart specific roles as a function of the various ways they are extracted, isolated, and proliferated (Barlow et al. 2008; Jansen et al. 2010; Kim et al. 2011; Dmitrieva et al. 2012; Strioga et al. 2012). Because of this, one tissue-derived MSC may be better suited for a specific therapy than another. Research in bone marrow-derived stem cells in horses established that these cells reach senescence at earlier passages than adipose- and umbilical cord-derived cells (Vidal et al. 2012).

Regardless of whether or not MSCs can differentiate into functional neurons, there is indication that MSCs promote neurogenesis after a stroke injury, but do not have a long-term survival after transplantation (Chen et al. 2003). Much like the neural stem cells, the benefits may arise from the production of neurotrophic factors and the modulation of vasculature observed equally from four different sources: bone marrow, adipose tissue, skeletal muscle, and myocardium (Lin et al. 2012).

Also similar to neural stem cells, the risk of mesenchymal stem cells developing into tumors must be considered, although only a few instances of tumor development have occurred subsequent to transplantation (Tolar et al. 2007) and specific derivations may have a greater propensity for tumorigenesis. For example, umbilical cord MSCs do not appear to develop into tumor progenitor cells in the presence of tumor cells, unlike bone marrow-derived mesenchymal stromal cells (Subramanian et al. 2012).

3.7 Extraembryonic Tissue Stem Cells

Regions of extraembryonic tissue stem cells include umbilical cord, placenta, amnion, and Wharton's jelly. As previously discussed, mesenchymal stromal cells may be the most popular for study, but amniotic epithelial cells, amnion-derived stem cells, placenta-derived stem cells, and umbilical cord matrix stem cells can also be found in extraembryonic tissue (Marcus and Woodbury 2008). Much like NSCs and MSCs, extraembryonic stem cells pertain to different germinal layers. The amniotic epithelium originates from the ectoderm, while the amnion-derived mesenchymal stromal cells are found in the mesodermal layer (Yu et al. 2009); thus, amnion-derived stem cells appear to contain a higher capacity for mesodermal cell lineages (Diaz-Prado et al. 2010). Amnion MSCs also exhibit less endothelial capabilities, further conferring potential embryonic specificity (Konig et al. 2012).

Given the specific germinal origin of these extraembryonic stem cells, there are discrete functionalities related to each cell type. Even with an increasing number of extraembryonic stem cell studies, there has been little yield demonstrating exactly

how these cells will function (Dobreva et al. 2010). Potential discrepancies have prompted the International Society for Cellular Therapy (ISCT) to define minimal criteria for definition of a stem cell as an MSC. Plastic adherence, cluster differentiation (CD) expression, and differentiation ability are some of the characteristics being considered (Dominici et al. 2006).

Furthermore, the Stem Cell Therapies as an Emerging Paradigm in Stroke (STEPS) was designed for the purpose of study interpretation in an attempt to standardize procedures (Borlongan et al. 2008; Borlongan 2009; Chopp et al. 2009). These guidelines are currently being adopted in neonatal hypoxic-ischemic injury and cerebral palsy (Borlongan and Weiss 2011).

Current studies with extraembryonic stem cells include transplantation of placenta-derived MSCs into animal models for stroke. In accordance with the proposed mode of action, these stem cells do not simply replace damaged cells; rather, they are observed to promote endogenous neurogenesis by fashioning a favorable microenvironment (Yarygin et al. 2009; Chen et al. 2012). Research with umbilical cord lining MSCs also demonstrates a greater immunosuppressive effect on the immune cascade and appears to have greater immunological immaturity than aged bone marrow MSCs (Deuse et al. 2011). An increase in leukemia inhibitory factor (LIF) by the umbilical cord lining MSCs may account for this potential (Najar et al. 2010).

3.8 Umbilical Cord Blood

Umbilical cord banking has become increasingly popular. With the possibility for both allogenic and autologous use, these stem cells could have vast therapeutic potential. Typically, umbilical cord blood refers to the mononuclear fraction, which includes hematopoietic progenitors, lymphocytes, monocytes, and MSCs. Despite its heterogeneity, these cells are considered immunologically immature. Accordingly, these cells are reported to modulate the immune response and reduce proinflammatory cytokine levels (Vendrame et al. 2005).

Transplantation of umbilical cord blood in animal stroke models has generated encouraging results. The optimal combination in favorable outcomes includes intravenous administration, 48 h post stroke, and a therapeutic dose of one million cells (Newcomb et al. 2006; Willing et al. 2003; Vendrame et al. 2004).

3.9 Adipose Tissue

In laboratory studies, treatment in adipose-derived stem cells has exhibited reduced infarct size, improved neurological function, and reduced level of cerebral inflammation and chronic degeneration in an intracerebral hemorrhage model (Kim et al. 2007; Leu et al. 2010). Despite these benefits in their use for stroke injury, side effects associated with adipose-derived stem cells have been noted. Initial research suggested that extensive passaging of adipose-derived stem cells might cause the

cell line to become spontaneously cancerous (Rubio et al. 2005), but this statement has since been retracted due to the inability to replicate the data (de la Fuente et al. 2010). Revisions to these studies have, however, demonstrated that adipose-derived stem cells can promote cancerous cells to produce tumors, but alone do not result in tumors (Ra et al. 2011). As in any stem cell type being translated for clinical applications, a careful analysis of risk-to-benefit ratio must be observed in order to advance a safe and effective cell therapy for stroke.

3.10 Induced Pluripotent Stem Cells

Stem cell differentiation was originally considered to be unidirectional, meaning, as the stem cell progresses through maturation, it becomes terminally differentiated and cannot regain multipotency. Recent experimentation is beginning to prove otherwise. Through transfection of specific transcription factors, embryonic-like stem cells can be regenerated from fibroblasts through retrograde manipulation. This transfection technique has also been applied to umbilical cord and placental MSCs, neural stem cells, and adipose-derived precursor cells to increase their potency (Cai et al. 2010; Tat et al. 2010).

A major benefit of retrograde conversion is the proliferation capacity of precursor cells. But the use of induced pluripotent stem cells appears to have some ramifications. As with many stem cells, both tumorigenesis and immunogenicity are of concern. The transfection technique used to generate precursor cells utilizes transcription factors of known oncogenicity. These cells, even when autologous, have also provoked an immune response leading to rejection (Zhao et al. 2011). Additionally, induced pluripotent cells are not as effective as embryonic stem cells (Liu et al. 2011). This was illustrated in a comparison study in which the induced pluripotent stems cells were less effective at generating human neurons in culture (Hu et al. 2010). Potential complications aside, induced pluripotent cells have been implicated in rat stroke models and yielded positive improvements in sensorimotor function (Jiang et al. 2011).

3.11 Menstrual Blood

The monthly cycling of the endometrial lining in the uterus has been a location of interest for researchers. Two different groups have isolated stem cells in this region, although it is unsure if they are the same cell line due to differences in culturing protocols (Meng et al. 2007; Patel et al. 2008). MenSCs (referred to as endometrial regenerative cells by the other group) exhibit multipotency. Hypoxic studies in neuronal cultures demonstrate menstrual blood stem cells provide protective benefits, an observation possibility related to the secretion of growth factors (Borlongan et al. 2010). These cells have also been implemented in middle cerebral artery occlusion (MCAO) rat studies, without immunosuppression (Borlongan et al. 2010; Allickson et al. 2011; Rodrigues et al. 2012).

3.12 Breast Milk

Although a study suggests these stem cells do not respond to normal culture protocol (Fan et al. 2010), different stem cell lines have been identified. Breast milk, as a source of stem cells, may provide advantages for harvesting, such as sparing women from invasive biopsies (McGregor and Rogo 2006). The nutritive components of breast milk have long been identified, but recently, investigation of vertical transmission from mother to offspring has included nonnutritive components, to elucidate the possibility of stem cell benefits (Meng et al. 2007). This source of stem cells also presents with the potential for autologous transplantation into women, similarly to menstrual blood.

3.13 Teeth

Teeth could prove to be a useful resource in harvesting stem cells in the future. As research accumulates, it appears as if stem cells can be obtained from teeth during various stages of their development (Miura et al. 2003). Teeth-derived stem cells have also demonstrated neurogenic potential (Zhang et al. 2009), possibly with greater aptitude than bone marrow-derived cells (Karaoz et al. 2011).

3.14 Embryonic Stem Cells

The preceding sections discussed adult stem cells. Another equally potential cell source for transplantation therapy in stroke is embryonic stem (ES) cells. Arguably, ES cells have been used as the yardstick of "stemness" properties, allowing an indefinite supply of stem cells which give rise to cells derived from all three germ layers. Indeed, ES cells transplanted in stroke animal models have been shown to contribute to repair neuronal (Hayashi et al. 2006) and vasculature damage (Oyamada et al. 2008) resulting in functional recovery (Yanagisawa et al. 2006; Wei et al. 2005; Pignataro et al. 2007; Theus et al. 2008; Yang et al. 2009). Additionally, the transplanted ES cells afford neurotrophic, angiogenic, and antiapoptotic effects (Wei et al. 2005; Pignataro et al. 2007; Theus et al. 2008; Yang et al. 2009; Li et al. 2008). Deposition of ES cells in brain and periphery following their transplantation in stroke animals has been visualized using imaging techniques (Hoehn et al. 2002; Lappalainen et al. 2008). Two major caveats that hinder the use of ES cells for transplantation therapy relate to the ethical concerns and risk of tumorigenicity.

3.15 Mode of Action

Although stem cells are transplanted, evidence suggests that the mode of action is not simply replacement of damaged cells. Since the stem cells do not remain in the brain, the mechanism is likely related to the release of growth factors that stimulate

endogenous stem cells. Stem cells may also furnish a cellular environment conducive for proliferation and survival through mediating inflammatory responses (Park et al. 2009; Sanberg et al. 2011, 2012). While we recognize the multiple therapeutic pathways involved in the functional recovery produced by cell therapy in stroke, the focus of this review is on highlighting the different origins of stem cells. Thus, we refer to the other book chapters dedicated to stem cell mechanisms of action.

3.16 Cotransplantation and Combination Therapy

As the evidence suggests, specific stem cells confer specific therapeutic potential. So there is consideration for treatment with multiple stem cell lines, simultaneously. There are reports of cotransplantation providing synergistic effects on stem cell survival. One such study combined the delivery of neural stem cells and adipose-derived stem cells, resulting in increased neural stem cell survival (Oh et al. 2011). Another study reports that cotransplantation of bone marrow stromal cells with embryonic stem cells ameliorated tumorigenesis (Matsuda et al. 2009). As related to stroke models, accompanying neural stem cells with epithelial cells heightened survival and differentiation (Nakagomi et al. 2009).

In addition to cotransplantation, combination therapy can incorporate a non-stem cell substrate to increase the efficacy of transplantation. Examples include combining bone marrow-derived stem cells with trophic factors to enhance survival and potentiation (Zhang et al. 2010) or providing a scaffold for stem cell adherence (Jin et al. 2010). There is still much to be developed by way of cotransplantation and combination therapy, but the possibility of enhanced stem cell benefits has been emergent thus far.

3.17 Other Factors

To date, many crucial variables persist with current stem cell techniques; many of these factors have recently been reviewed by us (Sanberg et al. 2012), factors such as optimal dose, route of administration, and sex of donor/recipient – all of which are likely to be dependent on the cell type being considered. We have investigated many of these undetermined parameters with umbilical cord blood for the conditions amyotrophic lateral sclerosis, Alzheimer's disease, and Sanfilippo syndrome (Sanberg et al. 2012); however, this information has yet to be resolved in regard to stroke.

Conclusions

Currently, there are an expanding number of sources available for stem cell harvesting, and as the data demonstrates, they may each impart their own benefits and limitations. General considerations include factors such as mode of action, immunogenicity, tumorigenicity, harvesting, proliferation capacity, and overall feasibility of use. These are all variables that must be addressed before

translational study. However, as the research continues to develop, so does the potential for the use of stem cells as a valuable treatment option for stroke.

Disclosure Cesario V. Borlongan and Paul R. Sanberg hold patents in stem cell technologies for the treatment of neurodegenerative disorders.

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Efficacy of Transplant and Endogenous Precursor and Stem Cell Interventions on Stroke Recovery: A Critical Assessment

4

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4.1 Introduction

In the last decade, there has been a growing realization that the injured brain has considerable capacity for reorganization and self-repair after injury. For example, brain damage results in increased dendritic growth and spine formation, synaptogenesis and upregulation of growth factors and reorganization of cortical sensorimotor maps (Jones and Schallert 1992; Cramer and Chopp 2000; Schallert et al.

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2000), processes implicated in brain repair and recovery. Importantly, similar forms of plasticity can be produced by behavioural experience, such as housing animals in enriched environments or exposing them to intensive rehabilitation, cortical stimulation or motor training (Ohlsson and Johansson 1995; Nudo et al. 1996; Kolb et al. 1998; Biernaskie and Corbett 2001; Kleim et al. 2003). Unfortunately, the extent of motor recovery after stroke remains limited with most patients experiencing debilitating deficits years after the injury (Cramer and Chopp 2000). Innovations in rehabilitation therapy suggested by clinical (e.g. constraint therapy). (Dromerick et al. 2000) and animal studies (Biernaskie and Corbett 2001) require intensive and prolonged periods of rehabilitation that may not be suitable for many stroke patients who may be constrained by other medical conditions (e.g. frailty), by motivational deficits or by cognitive dysfunction. While rehabilitation is helpful, many patients, particularly those with moderate to severe injury, are left with persistent impairments in daily living activities (Dobkin 2005). Clearly, additional interventions will be required to produce more complete recovery in most stroke patients.

One approach that has created a great deal of interest is to replace neurons lost as a result of stroke. The impetus for this notion is based on the discovery that the mammalian brain (including humans) produces new cells (neurogenesis) throughout life (Altman and Das 1965; Reynolds and Weiss 1992; Cameron et al. 1993). These newly generated cells proliferate in response to environmental stimuli and endogenous hormones (e.g. enriched environments, exercise) (Kempermann et al. 1997; van Praag et al. 1999; Shingo et al. 2003; Mak and Weiss 2010) and become functionally integrated into existing circuitry (Shors et al. 2001; van Praag et al. 2002), although evidence on this point is limited. Of particular relevance to stroke recovery is the observation that endogenous or transplanted stem cells *migrate* towards the site of damage thereby raising the exciting possibility that they may participate in functional recovery (Chen et al. 2001; Veizovic et al. 2001; Komitova et al. 2005). To date most studies have utilized exogenous delivery of neural precursor cells after focal ischaemia where early indications suggest that functional recovery is improved but the effects are small and limited in duration (Zhao et al. 2002; Bliss et al. 2006; Hicks et al. 2007; Hicks et al. 2009; Zhang et al. 2011); see Andrews et al. (2008). Similar results have been achieved in a few studies where growth factors or environmental enrichment has been used to encourage survival and migration of endogenous precursor cells to the peri-infarct cortex (Komitova et al. 2005; Tsai et al. 2006; Kolb et al. 2007). In these studies, it is unclear whether the newly formed cells are being incorporated into existing circuits or whether the cells are fully functional. It is possible that the observed benefits may be due to indirect effects of these cells (e.g. source of growth factors) on undamaged circuits that ultimately reorganize in response to the injury (Bliss et al. 2007).

In this chapter, we attempt to critically assess both endogenous and exogenous approaches to repair the stroke-damaged brain with special emphasis on additional factors including behavioural outcome measures, post-stroke environment, age and other variables that modulate “apparent efficacy” of stem cell treatments.

4.2 Mobilization of Endogenous Stem Cells and Stroke Recovery

The existence of neural stem cells in the adult brain holds great promise for the development of neural repair strategies (Reynolds and Weiss 1992; Weiss et al. 1996). Neural stem cells and their direct progeny (progenitor cells) – collectively known as neural precursor cells (NPCs) – possess the ability to generate all the neural cell types that comprise the central nervous system and are therefore thought to be good targets for the development of cell-based therapies to repair the injured CNS. Since their original isolation and characterization in vitro, much has been learned about the in vivo location (the neural stem cell niche) and the factors that regulate the behaviour and lineage dynamics of resident NPCs. The prospect of activating these endogenous NPCs using biologics and enticing them to contribute to repair of the injured brain has become a compelling prospect. There are a number of advantages of manipulating endogenous precursors in situ including the lack of immune rejection issues and the circumvention of concerns of altering the growth characteristics of cells (i.e. generating transformed cell lines) with prolonged culture prior to their use in transplantation paradigms (Morshead et al. 2002).

Adult NPCs can be found along the entire neuraxis of the adult CNS (Reynolds and Weiss 1992; Weiss et al. 1996) and have been well studied in the two neurogenic regions that persist into adulthood: the subventricular zone (SVZ) and the dentate gyrus (DG) of the hippocampus (Morshead et al. 1994; Palmer et al. 1995). The SVZ lines the walls of forebrain lateral ventricles and is comprised of a single cell layer of ependymal cells and an adjacent 2–4-cell-layer-thick region called the subependyma (Morshead et al. 1994; Chiasson et al. 1999). Neural stem cells in the subependyma of the SVZ are slowly proliferating cells (Morshead et al. 1994) that express glial fibrillary acidic protein (GFAP) (Doetsch et al. 1999; Morshead et al. 2003; Garcia et al. 2004). The rapidly dividing progeny undergo cell death or migrate along the rostral migratory stream to the olfactory bulb, where they differentiate into interneurons and become functionally integrated into the neuronal network (Lois and Alvarez-Buylla 1994; Morshead et al. 1998; Carleton et al. 2003). Neurogenesis in the rodent olfactory bulb is thought to play a role in olfactory learning and memory (Krakauer et al. 2012). The adult human SVZ similarly contains a population of GFAP-expressing cells which are capable of forming self-renewing, multipotent colonies when isolated in vitro, thereby confirming the presence of NPCs in the adult human brain (Sanai et al. 2004; van den Berge et al. 2010). Furthermore, migrating neurogenic progeny have been observed in the human brain albeit in much smaller numbers than what is observed in rodents (Curtis et al. 2007; Wang et al. 2011).

Similarly, within the DG of the hippocampus, a subpopulation of GFAP-expressing cells with a radial morphology has been purported to act as multipotent self-renewing neural stem cells (Palmer et al. 1997). While the existence of neural stem cells within the hippocampus has been challenged on more than one occasion (Palmer et al. 1997; Seaberg and van der Kooy 2002; Suh et al. 2007), it is unrefuted that the hippocampus is a neurogenic region of the brain throughout life and the

newly born neurons generated within this region play a role in hippocampal plasticity and cognitive function (Jessberger and Gage 2008).

NPCs in the adult brain elicit the fundamental properties in vivo that would benefit the development of strategies to promote their contribution to neural repair, namely, proliferation, migration and differentiation into neural phenotypes. Indeed, it has been demonstrated that brain injury alone can activate endogenous NPCs (Liu et al. 1998; Jin et al. 2000; Parent et al. 2002; Zhang et al. 2004; Kernie and Parent 2010). The activation of endogenous NPCs in response to stroke has been demonstrated using proliferation assays (i.e. measuring the numbers of BrdU-labelled cells in the SVZ and DG) as well as in vitro colony-forming assays. These assays reveal that injury alone generates a larger pool of cells that can potentially be utilized for brain repair.

The first demonstration of the recruitment of NPCs directly to the stroke-injury site was in a focal model of transient middle cerebral artery occlusion (MCAo) in rats. Immunohistochemistry revealed a population of SVZ-derived NPCs proliferating, migrating to the site of infarct and generating new neurons in the striatum and parietal lobe (Arvidsson et al. 2002; Parent et al. 2002). The number of striatal neurons generated was small, and functional recovery was not assayed, but nonetheless, these studies demonstrated the brain's inherent, albeit limited, capacity for self-repair. Further studies suggested that this activation and recruitment process persists for several months after the ischaemic attack (Thored et al. 2006; Yamashita et al. 2006) and that newly formed neurons become synaptically integrated as determined by morphological and electrophysiological studies (Yamashita et al. 2006; Hou et al. 2008). These phenomena have more recently been examined in humans, where post-mortem biopsies of stroke patients have shown the presence of proliferating and differentiating cells in the ischaemic penumbra as well as the ipsilateral SVZ (Jin et al. 2006; Marti-Fabregas et al. 2010). However, the degree of endogenous activation following stroke is clearly not sufficient for functional recovery as demonstrated by the persistent functional impairments observed in patients following stroke.

With the goal of augmenting the self-repair mechanisms of the brain, a thorough understanding of mechanisms that underlie the activation of NPCs is needed. Various NPC "activation factors" have been examined for their potential role in modifying NPC proliferation kinetics, increasing NPC cell survival, enhancing cell migration to infarct site and/or promoting neurogenesis and angiogenesis. Factors such as vascular endothelial growth factor (VEGF) (Jin et al. 2000; Wittko et al. 2009; Shin et al. 2010), epidermal growth factor (EGF) (Craig et al. 1996), fibroblast growth factor (FGF) (Kuhn et al. 1997) (Leker et al. 2007), transforming growth factor alpha (TGF-α) (Guerra-Crespo et al. 2009) and erythropoietin (EPO) (Wang et al. 2012) have been shown to influence the proliferation and/or differentiation of endogenous NPCs in the adult brain under baseline conditions and following stroke. Craig and colleagues (1996) were the first to demonstrate that the intraventricular administration of exogenous EGF expanded the SVZ progenitor population in vivo and induced their migration away from the neurogenic niche lining the lateral ventricles and into the surrounding parenchyma in the intact adult brain.

This demonstration that endogenous NPCs could be modified by the application of exogenous factors led to a number of factors, alone and in combination, being utilized in stroke models. In addition to those mentioned above, glia-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), granulocyte colony-stimulating factor (G-CSF), stem cell factor (SCF) and human chorionic gonadotropin (hCG) (Teramoto et al. 2003; Leker et al. 2007; Kobayashi et al. 2006; Schabitz et al. 2007; Kolb et al. 2007; Belayev et al. 2009) have been administered in stroke models.

With the goal of clinical application, enhancing NPC proliferation using mitogens may not be the most appropriate avenue for increasing the size of the NPC pool due to the risk of tumorigenesis that is implicit with enhanced proliferation. An alternative approach to expanding the NPC pool post-ischaemia is by promoting cell survival through inhibition of apoptosis. Recently it was reported that cyclosporin A (CsA), a commonly used immunosuppressive drug, acts directly on NPCs to increase their survival without affecting cell cycle kinetics (Hunt et al. 2010). While the pro-survival mechanism is not clear, the administration of CsA in vivo results in a >2-fold increase in the size of the NPC pool in control mice. Moreover, mice that received CsA following stroke showed an expansion of the NPC pool, migration of the NPCs to the site of injury, new tissue formation at the site of cortical ischaemia, as well as recovery of motor function (Erlandsson et al. 2011). Regulating the mode of division of NPCs to promote symmetry of division at the expense of asymmetric division will also result in increased numbers of NPCs in vivo. Signalling molecules such as Notch (Wang et al. 2009) and Wnt (Piccin and Morshead 2011) have been shown to increase in models of stroke and during tissue regeneration in the adult brain. Moreover, intraventricular infusion of Notch activators leads to improved motor function in stroke-injured mice (Androutsellis-Theotokis et al. 2006; Wang et al. 2009). These studies highlight the importance of considering a number of different targets to increase the size of the NPC pool for application in endogenous repair strategies.

The redirected cell migration towards the site of injury has been shown to be regulated by chemoattractrant molecules such as stromal cell-derived factor (SDF-1), monocyte chemoattractant protein (MCP-1), angiopoietin (Ang-1), slits, matrix metalloproteases, galectin-1 and osteopontin (Imitola et al. 2004; Thored et al. 2006; Yamashita et al. 2006; Langdon and Corbett 2012; Kempermann 2011). Thin astrocytic processes and blood vessels have been shown to serve as scaffolds for the migration (Yamashita et al. 2006). Understanding the factors that promote migration and the cells that respond to injury will facilitate the design of future therapies to enhance the regenerative process after stroke.

Functional benefits have been achieved by mobilizing endogenous stem cells with growth factors or combinations of growth factors, yet many basic issues remain to be resolved. The challenge of inducing effective functional integration of newly generated neurons into existing neural and synaptic networks is ongoing, and reports to this effect remain controversial. It has been suggested that substantial replacement of infarcted tissue by NPCs is unlikely and that NPC-mediated neurogenesis may be inconsequential in functional recovery (Kempermann 2011). Hence, it is not

clear that developing self-repair strategies following stroke should be restricted to neurogenesis as reconstruction of glial cells and the vascular system is also required. Notably, deleterious effects of enhanced neurogenesis have been reported (Scharfman and Hen 2007), highlighting the importance of balancing cell replacement with positive outcomes.

4.3 Transplant Approaches to Stroke Recovery

A variety of different cell types (hematopoietic, immortalized cell lines, neural stem/precursor cells.) and routes of delivery (e.g. intravenous, intracerebral) have been used in transplant studies. Since these topics are covered in detail by other authors in this book, they will not be the focus here. Instead, we will direct discussion towards the efficacy of different stem cell approaches to improving functional recovery following stroke with a critical assessment of the associated outcome measures employed. This is predicated on the widespread failure of stroke neuroprotective strategies in which preclinical studies failed to adequately incorporate fundamentally important aspects of human stroke (Corbett and Nurse 1998; Endres et al. 2008) thereby resulting in what has been termed translational roadblock (Endres et al. 2008). Further, we will not consider studies where the goal was to achieve neuroprotection for the obvious reason that the time window, like for tissue plasminogen factor, is very narrow with the result that only a minority of stroke patients (~10 %) would derive benefit.

A number of studies have used systemic delivery of stem cells with the intravenous route offering the least risk for ultimate use in humans. In one early study, Chopp and colleagues subjected rats to 2 h of transient MCAo using the intraluminal suture method (Li et al. 2001). Ischaemia was followed 24 h later by intracarotid delivery of mesenchymal stem and progenitor cells (MSC). Animals were then tested several times over the 14-day post-stroke survival period using a modified neurological test score and an adhesive tape removal test (Schallert et al. 1982). Rats receiving MSC recovered more rapidly compared to ischaemic controls, but both groups improved by the final day 14 behavioural test. Results were similar using an intravenous delivery route 24 h after MCAo (Chen et al. 2001) with marginally faster recovery in neurological deficit scores and tape removal tasks. Interestingly, transplants of conditionally immortalized neuroepithelial cells into the *undamaged* hemisphere several weeks after 60 min of MCAo improved performance on the tape removal test and mitigated drug-induced rotational asymmetry (Veizovic et al. 2001). A minority of the transplanted cells crossed the midline and migrated to the peri-infarct zone suggesting that the benefit was perhaps due to secondary effects (e.g. reduction in atrophy) in the undamaged hemisphere. Complicating the interpretation is the finding that infarct volumes were reduced in transplanted animals suggesting these animals may have had smaller infarcts to start with, which would account for the perceived modest functional benefits of the transplants. This possibility is further supported by the fact that there is a sensitive time window of approximately 1 month following stroke when recovery-promoting interventions are most

effective (Biernaskie et al. 2004; Murphy and Corbett 2009) and the transplants in this study were administered when this window is closing. Using human hNT cells (derived from embryonic carcinoma cells) transplanted 1 week after a permanent distal MCAo, Bliss and colleagues found good survival (~40 %) after 4 weeks, but this did not translate into very robust post-stroke behavioural recovery (Bliss et al. 2006). Indeed, the only improvement was on a ledged beam test with no effect of transplantation on forelimb asymmetry (i.e. cylinder and tape removal tests). In a subsequent study, this group determined that transplanted embryonic human neural stem cells in nude, T-cell-deficient rats produced neurological improvement in a limb-placing task. This effect was attributed to cell-induced improvement of blood-brain barrier integrity, reduced inflammation and enhanced vascularization that was shown to be VEGF dependent (Horie et al. 2011). Surprisingly, recovery of limb placing varied considerably among cell-treated animals, with some showing marked improvement in limb placing 1 week after transplantation, while remaining rats took several weeks longer to recover. It is unclear what accounts for differences in recovery (e.g. variation in stroke size, location). The effects of human NPCs on stroke recovery were examined in another series of experiments that included additional behavioural tests (postural reflexes, cylinder test and body swing test); however, in this study, there was no early improvement in recovery even on the limb-placing task with significant benefits only becoming apparent 4 or more weeks after stroke depending on the test (Andres et al. 2011). In contrast, administration of human umbilical tissue-derived cells early (1 day) or up to 30 days post-stroke improved neurological deficit scores and decreased tape removal latencies in rats subjected to 2 h of MCAo (Zhang et al. 2011). This recovery was associated with enhanced synaptogenesis, neurogenesis and angiogenesis.

In summary, a variety of stem cell types have been used in efforts to promote functional recovery after stroke. Results appear encouraging particularly since efficacy has been demonstrated in different species (rat, mouse) and stroke models (e.g. transient and permanent MCAo); however, the magnitude of the enhanced recovery remains rather modest in all of these studies, suggesting that additional ways to enhance the activity and survival of transplanted and resident stem and NPCs may be required.

4.4 Use of Biotechnology to Enhance Stem Cell Therapies

Stem cell therapies for stroke have been pursued by two fundamentally different approaches: (1) stem cell transplantation and (2) endogenous stem cell stimulation. The former includes multiple different types of stem cells, such as adult NPCs, embryonic stem cells and mesenchymal stem cells, among others. The sites for transplantation include direct injection into the brain (Daadi et al. 2008) or systemic injection into the blood (Wang et al. 2008). These strategies have met with some success (Bliss et al. 2007) and are discussed here and in other chapters. Herein, our focus is on endogenous stem cell stimulation although these technologies can also be utilized in transplant studies with or without mobilization of endogenous cells.

The challenge in endogenous stimulation is how to stimulate NPCs specifically without stimulating other cells types. One way to achieve this is with a local or targeted delivery strategy of activation factors (Lanfranconi et al. 2011).

The blood-brain barrier makes local delivery strategies difficult because it significantly reduces (or inhibits) the diffusion of biomolecules across the vasculature into the brain (Pardridge et al. 1992). Thus, the blood-brain barrier (BBB) makes normal routes of delivery, such as oral or intravenous (i.v.), ineffective (Ferber 2007). While some molecules, such as erythropoietin (EPO), can cross the BBB (Hanson and Frey 2008), large systemic doses are required to achieve the local concentration in the brain tissue required for efficacy, resulting in blood thickening systemically which is undesirable, especially in cases of stroke (Torup 2007).

There are some strategies that are being investigated to promote greater accumulation in the brain after systemic i.v. delivery. For example, there are some formulations that have either the biomolecule modified with poly(ethylene glycol) (PEG) (Meinel et al. 2004) or nano-/microparticle (in which the biomolecule is encapsulated) modified with PEG (Li et al. 2011). Modification with PEG prolongs blood circulation time and increased accumulation in the brain; however, the penetration distance into the brain, the accumulated dose in the brain and the associated systemic toxicity can be limiting.

Alternative strategies include local delivery strategies, such as focal opening of the BBB with, for example, ultrasound (de Boer and Gaillard 2007). In this strategy, ultrasound is focused in a specific region of the brain and opens the BBB for a defined period of time. The advantage of this technique is the precise location of the BBB opening. The disadvantage is that all circulating molecules and cells can now cross the BBB in that defined volume of tissue.

Another strategy includes use of intraventricular infusion of biomolecules (Jonhagen et al. 1998). Here, a catheter/minipump system is employed where a cannula is inserted through the brain into the ventricle. Since the endogenous stem cells line the lateral ventricles, this strategy is effective for their stimulation (Kolb et al. 2007). A potential limitation of this strategy is the brain tissue damage associated with cannula insertion. Moreover, the biomolecules delivered to the cerebrospinal fluid in the ventricles are dispersed throughout the brain and spinal cord. Consequently, limited amounts of these factors diffuse into the brain tissue.

A new strategy has recently been proposed wherein biomolecules are dispersed within a hydrogel that is injected directly on the brain tissue (Cooke et al. 2011; Wang et al. 2012) In this example, a small burr hole is made in the skull and the dura is pierced, into which a small volume of hydrogel is injected, thereby releasing biomolecules directly to the brain. In this strategy, the blood-brain barrier is circumvented and the biomolecules are released into the tissue. The advantage of this technique is the local and targeted strategy achieves high local concentration of biomolecules directly into brain tissue, for endogenous tissue stimulation. The limitation of this technique is that biomolecules have to diffuse through brain tissue in order to reach the endogenous cells. While the distance required to reach the NPCs lining the lateral ventricles has been achieved in a mouse brain, it is not clear whether the same success will be realized in larger animal models, including in the human brain.

4.5 Is Stem Cell Therapy for Stroke Ready for Prime Time?

In the 1990s, it appeared that there were an abundance of new drugs, all with the potential to markedly reduce stroke damage with a resultant reduction in functional impairments. Dozens of putative neuroprotective agents were rushed into clinical trials but all failed (O'Collins et al. 2006). What went wrong? Consensus was that both preclinical (i.e. animal models) and clinical studies were seriously flawed (Endres et al. 2008). In order to not repeat the same mistakes, there have been two round-table meetings of scientists, clinicians and industry representatives to establish guidelines and recommendations for Stem Cell Therapies as an Emerging Paradigm for Stroke (STEPS I and II) (Wechsler 2009; Savitz et al. 2011). One issue is whether existing stem cell studies measure up to exiting STEPS guidelines and a second is whether the STEPS II guidelines go far enough to ensure that we do not generate another translational roadblock as with neuroprotection.

A key consideration in stroke recovery is the outcome measures used. As outlined in STEPS II (Savitz et al. 2011), a battery of tests, sensitive to the deficits, carried out at intervals over at least a month should be employed, preferably in multiple laboratories. Only some of these criteria have been met. Most studies use several behavioural tests conducted for 2 or more weeks after the stem cell interventions (Li et al. 2001; Veizovic et al. 2001; Modo et al. 2002; Zhao et al. 2002; Bliss et al. 2006; Andres et al. 2011; Zhang et al. 2011). However, the behavioural tests are often based on subjective neurological deficit scores, with some using tape removal and/or cylinder tests to gauge sensory-motor impairments (Bliss et al. 2006; Andres et al. 2011; Zhang et al. 2011). The problem with such tests is that they reveal deficits early after stroke, but often, there is near complete spontaneous recovery after several weeks (Murphy and Corbett 2009). Indeed, it appears that in many cases, the stem cell treatments are accelerating recovery since the control animals are showing the same recovery profiles albeit at a slightly slower rate. These findings are reminiscent of early work with amphetamine (Feeney et al. 1982) where this drug sped up recovery on simple beam-traversing tasks and attempts to demonstrate clinical efficacy have failed (Gladstone et al. 2006). Because of the inherent risks and invasive nature of stem cell therapies, they would only be used on patients with chronic, debilitating deficits that accompany moderate to severe stroke. Accordingly, animal models investigating the efficacy of endogenous or transplant stem cell approaches need to incorporate impairments that are not only chronic but that target common clinical disabilities such as the inability to use the fingers. Only a few stem cell studies have used more sensitive sensory-motor reaching tasks such as the staircase test or single pellet reaching tests that assess skilled use of the digits (Kolb et al. 2007; Andrews et al. 2008; Liu et al. 2011). Using human adult bone marrow-derived cells, Andrews and colleagues (Andrews et al. 2008) reported substantial recovery in a single pellet reaching task compared to control rats that correlated with sprouting of the corticorubral tract into the damaged hemisphere. In our own studies, using cortical and striatal transplants of mouse SVZ cells into rats with forelimb motor cortex stroke, we detected a small (~10 %), nonsignificant improvement in staircase skilled reaching compared to controls. In another study, transplants of human NPCs

in rats exposed to enriched environments produced improvement in spontaneous limb use in the cylinder test, but there was no benefit in resolving reaching impairments in the staircase test (Hicks et al. 2009). The general limited recovery of skilled reaching compared to the uniform benefit in resolving simple neurological deficits highlights the need to use more sensitive and clinically relevant outcome measures in animal studies.

Another important consideration is that stroke patients typically receive rehabilitation that exerts its recovery-enhancing effects by promoting the same neuroplasticity processes (e.g. upregulation of growth factors, sprouting of dendritic spines and new connections) (Murphy and Corbett 2009) that are activated by stem cell interventions (Andres et al. 2011). Thus, it is important that preclinical studies incorporate rehabilitation, along with other key attributes of human stroke (e.g. older animals, disease co-morbidity) since these variables can markedly alter the efficacy of stem cell treatments. For example, rehabilitation affects the behaviour of transplanted stem cells. Hicks showed that transplanted mouse NPCs derived from SVZ migrated greater distances and showed a trend towards increased survival in rats that were exposed to a combination of enriched environments and exercise following focal ischaemic stroke (Hicks et al. 2007). In more recent work, we found that EGF- and EPO-induced mobilization of the endogenous NPC pool resulted in significantly faster recovery of skilled reaching after cortical stroke in rats provided it was *combined* with enriched rehabilitation. This is the first demonstration that stem cell therapy produces an additive benefit in stroke recovery *above* what can be achieved with an optimized rehabilitation paradigm. In the absence of such demonstration, stem cell therapies should not be advanced to clinical trials due to the inherent risk and invasiveness to patients that are predominantly elderly and frail.

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Part II

Translational Imaging Techniques to Study Cell Therapy

Cell Labeling Methods for Noninvasive MR Imaging of Stem Cells

5

Jeff W.M. Bulte

5.1 Introduction

Cellular imaging or cell tracking using magnetic resonance imaging (MRI) has now entered the clinic, and there is a strong consensus in the field that this will eventually be an integral part of evaluating novel experimental stem cell therapies aimed at treating ischemic events. Major clinical questions need to be addressed in order to ensure that cell therapy can be performed successfully in a more mainstream setting beyond a few academic centers where everything is taking place now. Ideally, clinical cell tracking should address the following key questions: (1) Are the cells delivered/injected correctly? (2) How many stem cells have been correctly delivered/homed into the stroked brain? (3) For how long do these stem cells survive? (4) Do they replicate following administration including the formation of unwanted neoplasms? (5) Do stem cells differentiate in vivo, and if so, when does this occur? (6) What are the morphological, physiological, or functional changes of the brain that are indicative of therapeutic success or failure? At the present time, unfortunately, there is no single imaging technique that can address all the questions.

Except for fluorine imaging (see below), MRI cell tracking is an indirect technique for detecting cells based on loading cells with contrast agents or transfecting cells with reporter genes that affect the water signal. In this chapter, several methods that can be used to tag cells are outlined, with a discussion of some of the advantages and limitations for each method.

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5.2 Cell Labeling with Paramagnetic Agents

Paramagnetic metals include the lanthanide and rare Earth metals gadolinium Gd^{3+} and manganese Mn^{2+}, chelated to prevent metal toxicity. Because of their low relaxivities (efficacy of affecting the water signal per unit concentration of metal), a high intracellular accumulation is required. At lower fields, these contrast agents make the cells appear as hyperintense. The evaluation as cellular labeling agents is usually performed by measuring the relaxivity of the paramagnetic chelates in solution, which often is not representative of the contrast of labeled cells once transplanted in vivo. Intracellular clustering and limited access to diffusing water protons limit the inner sphere relaxivity, leaving the magnetic susceptibility effect as predominant mechanism of contrast. In such cases, the cells will appear hypointense, in particular when using higher field strengths and more T2-weighted pulse sequences. Most labeling protocols have relied on simple pinocytosis of the paramagnetic contrast agent added to the medium or cell solution (Aoki et al. 2006; Crich et al. 2004). In contrast to the endosomal distribution of Gd chelates after pinocytosis, electroporation can result in intracellular diffusion of Gd molecules and a greater T1 relaxivity and higher MR signal intensity of labeled cells (Terreno et al. 2006).

For the use of paramagnetic metals, the potential for metal dechelation has been a grave concern. In particular, when internalized into endosomes that fuse with lysosomes, lowering the pH, dechelation over time will occur even for the macrocyclic chelates such as DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) and its derivatives. Some contrast agent manufacturers are now being sued for large sums of money for causing gadolinium chelates-induced death in patients with impaired renal function. Patients with nephrogenic systemic fibrosis (NSF) are unable to clear gadolinium chelates out of the body fast enough and have shown what detrimental effects gadolinium can have. Therefore, MRI cell tracking using paramagnetic agents has not been pursued in the clinic and is not likely going to occur anytime soon.

5.3 Cell Labeling with Superparamagnetic Iron Oxide (SPIO) Agents

5.3.1 Simple Incubation

Certain cell types, such as monocytes, macrophages, and mesenchymal stem cells (MSCs), have the natural ability to phagocytose magnetic nanoparticles. This includes the larger magnetic micro-sized-particles that are coated with polystyrene (Hinds et al. 2003). Because of the chemical makeup of the coat and the fact that some manufacturers explicitly state that the material does not have a guaranteed sterility, their clinical utility is limited. Smaller, (carboxy)dextran-coated SPIOs including the former available clinical products Feridex® or Endorem® and Resovist® have been used for phagocytic stem cell labeling without additional

modification, but for an efficient cell labeling of most cell types, they need to be complexed with transfection agents.

5.3.2 Transfection Agent-Mediated SPIO Labeling

Since transfection agents have been used for long periods of time to shuttle macromolecules (i.e., DNA and plasmids) into cells, it was postulated that by coating the surface of SPIO with a transfection agent, an increased intracellular labeling could be obtained. The first such example was the use of magnetodendrimers that can label stem cells by simple incubation at low concentrations (Bulte et al. 2001). Subsequently, commercially available transfection agents were used with similar results (Frank et al. 2003). Transfection agent-mediated SPIO labeling is now the most commonly used procedure by many labs around the world. Recently, ferumoxytol (Feraheme®) SPIO particles have been conjugated (Chen et al. 2011) or complexed (Thu et al. 2012) to protamine sulfate, with the two separate components being clinically used (Figs. 5.1 and 5.2), and hence, their future clinical use for MRI cell tracking is anticipated.

5.3.3 Viral Shell-Mediated SPIO Labeling

Viruses have coats that bind specifically to the membrane of mammalian cells. They often are composed of glycoproteins resembling lectins. Reconstituted Sendai virus envelopes have been used in one of the earliest studies on intracellular SPIO labeling of peripheral blood mononuclear cells and fetal neural cells (Bulte et al. 1993; Hawrylak et al. 1993). Later studies have used hemagglutination viruses, also from Asian origin, with superior labeling but limited availability (Toyoda et al. 2004). The host immune response toward these foreign antigens is not known, and the materials are not available commercially.

5.3.4 Electroporation and Sonoporation

Before transfection agents were used as a means to shuttle DNA into cells, electroporation was widely used to mechanically induce small membrane openings that enabled passage of large molecules. It is still used for hard-to-transfect cells (such as embryonic stem cells), but nuclear transfection has now largely been replaced by the use of transfection agents. One reason is that the genetic material has a tendency to remain trapped within endosomes in the cytoplasm. Electroporation was reintroduced as a method to achieve intracellular endosomal SPIO labeling (Walczak et al. 2005), and it can label cells with particles that otherwise need to be chemically modified for cell labeling, e.g., manganese oxide (Gilad et al. 2008). Subsequently, the electroporation technique was extended to sonoporation, which uses ultrasonic waves to induce similar membrane openings (Figs. 5.3 and 5.4) (Qiu et al. 2010; Xie et al. 2010).

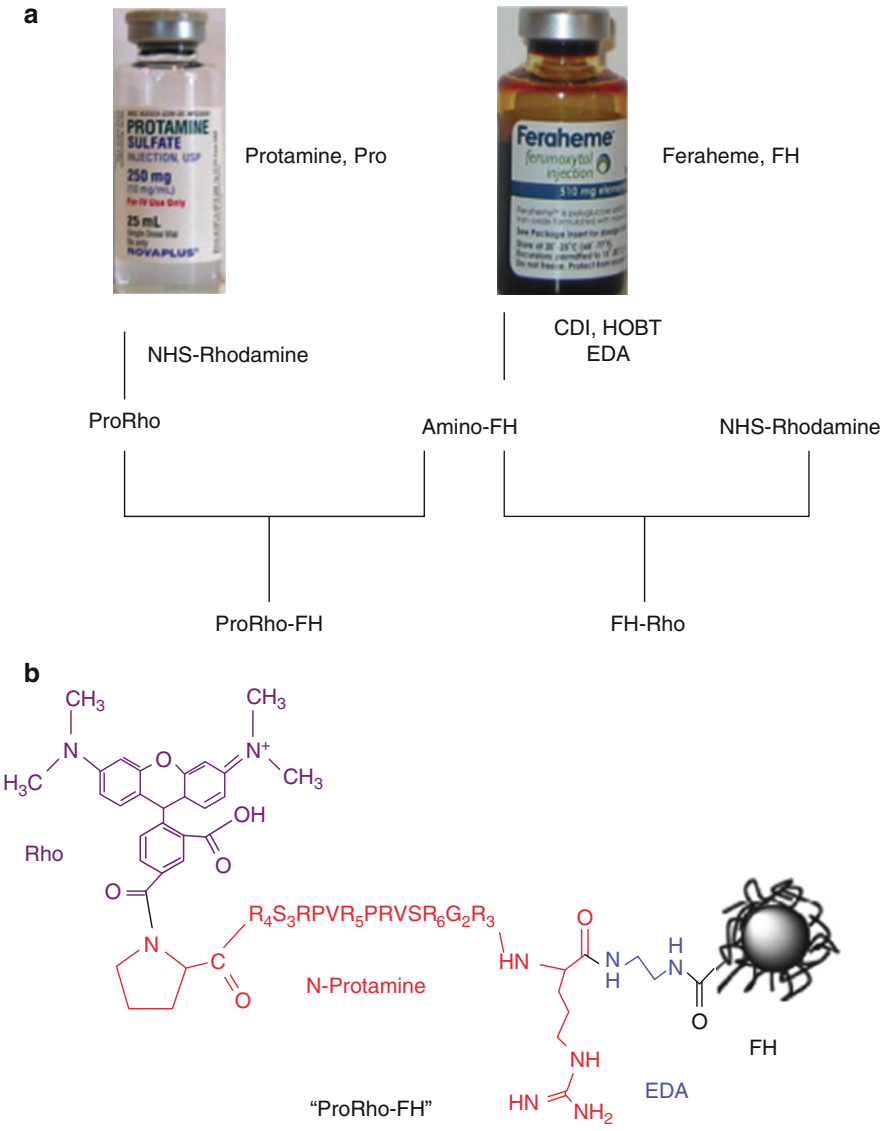

Fig. 5.1 Synthesis of a magnetofluorescent nanoparticle, ProRho-FH, using protamine (Pro) and Feraheme® (FH). (**a**) Synthesis of ProRho-FH. *CDI* carbodiimide, *EDA* ethylene diamine, *HOBT* hydroxybenzotriazole. (**b**) ProRho-FH design (Reproduced, with permission, from Chen et al. (2011))

5.4 Cell Labeling with Fluorinated Compounds

Fluorine MRI was introduced shortly after proton MRI (Holland et al. 1977), and it was immediately realized that it represents a true tracer technique as there is no

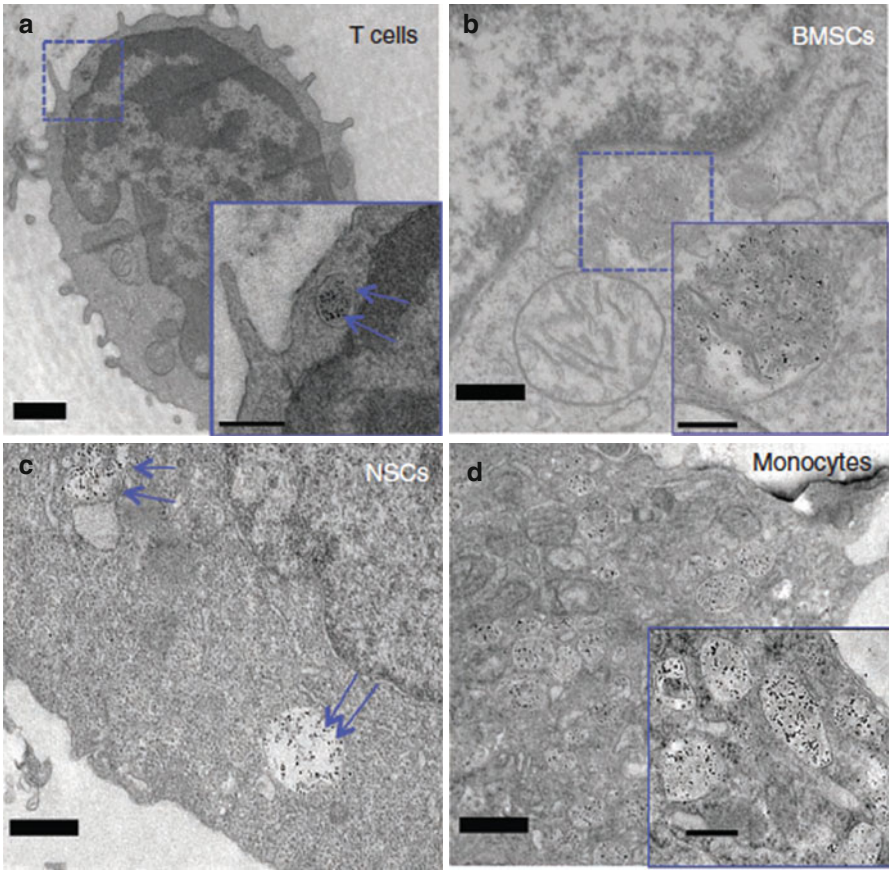

Fig. 5.2 Internalization and encapsulation of heparin-protamine-Feraheme® (HPF) nanocomplexes in HPF-labeled cells (**a**–**d**). Transmission electron microscopy of HPF-labeled T cells (**a**), HPF-labeled bone marrow stem cells (**b**), HPF-labeled neural stem cells (**c**) and HPF-labeled monocytes (**d**) shows that HPF nanocomplexes were encapsulated in endosomes as electron-dense nanoparticles (indicated by *blue arrows* in (**a** and **c**), and shown in *insets* in (**a**, **b**, and **d**)). *Black scale* bar in (**a**), 0.5 μm. *Black scale* bar in (**b**), 0.4 μm. *Black scale* bars in (**c** and **d**), 0.6 μm. *Inset scale* bars in (**a** and **d**), 0.3 μm. *Inset scale* bar in (**b**), 0.1 μm (Reproduced, with permission, from Thu et al. (2012))

endogenous background signal. ^{19}F "hot spot" MRI cell tracking was introduced in 2005 (Ahrens et al. 2005) using a similar approach for cell labeling as that most commonly used for SPIO labeling, e.g., coating the ^{19}F emulsions with commercially available transfection agents. Modifications of perfluorocarbons as cell labeling agents have been made to avoid the use of transfection agents, e.g., endowing them with a cationic surface (Fig. 5.5) (Ruiz-Cabello et al. 2008). One of the main advantages of ^{19}F MRI cell tracking is that quantification of the label in tissues is straightforward, allowing one to perform in vivo "cytometry" (Srinivas et al. 2009).

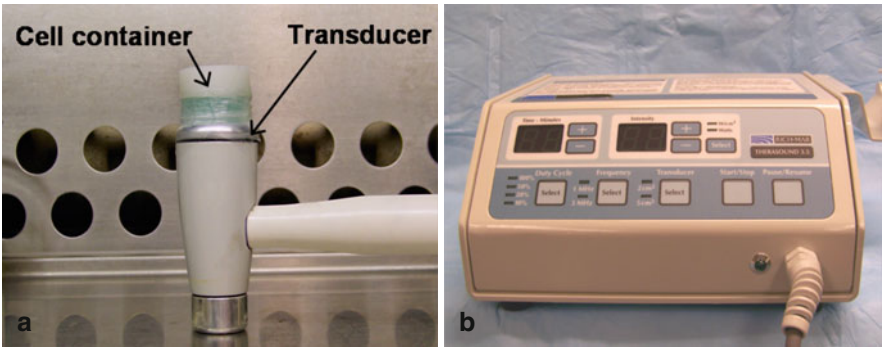

Fig. 5.3 Sonoporation apparatus for cell labeling. (**a**) The cell labeling container, a sterilized plastic tube, is placed on the top of the ultrasound transducer. (**b**) The transducer is connected to a therapeutic ultrasound generator, which has a digital panel for the adjusting the output of ultrasonic energy (Reproduced, with permission, from Qiu et al. (2010))

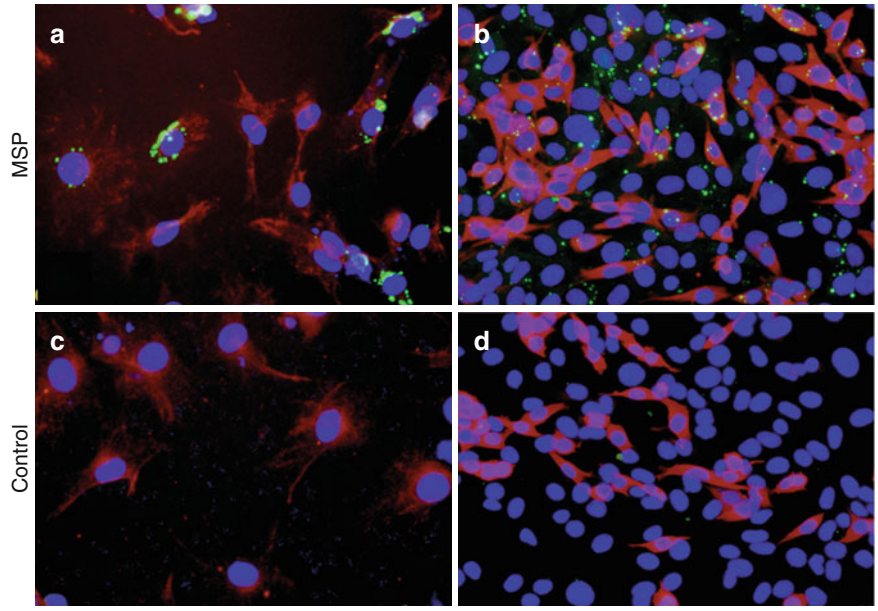

Fig. 5.4 Stem cell differentiation after sonoporation with Feridex®. (**a**–**d**) Neural stem cells develop typical neuronal morphology and express the proteins nestin and β-tubulin (*pink*) for both sonoporated (**a**, **b**) and control cells (**c**, **d**). Dextran (*green*)-containing endosomes in sonoporated cells can be detected (**a**, **b**) with proliferation of neural stem cells and dilution of Feridex® before terminal differentiation, which is absent in the control cell groups (**c**, **d**) (Reproduced, with permission, from Qiu et al. (2010))

5.5 Labeling with Reporter Genes

Neither paramagnetic, SPIO, nor perfluorocarbon-based labeling can be used for tracking of cell survival or stem cell differentiation into downstream lineages.

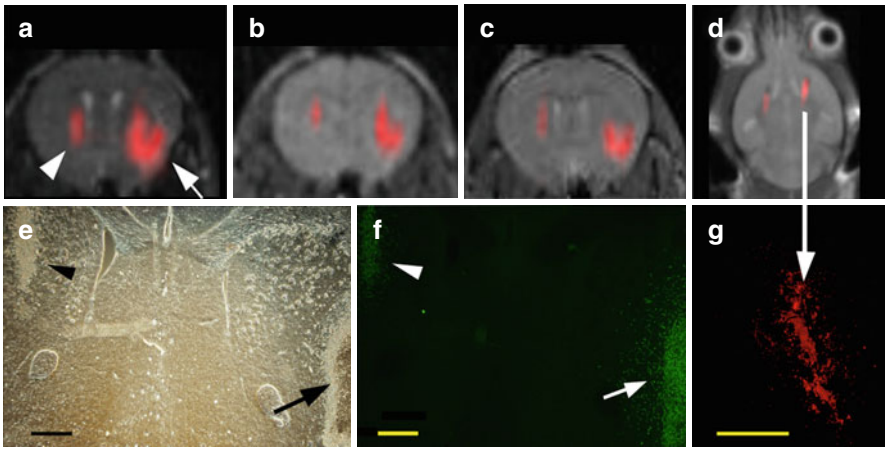

Fig. 5.5 In vivo MR imaging of transplanted neural stem cells, with the ^{19}F signal superimposed on the ^{1}H MR images. Shown are MR images at 1 h (**a**), 3 days (**b**), and 7 days (**c**) after injection of 4×10^4 (left hemisphere, *arrowhead* in **a**) or 3×10^5 (right hemisphere, *arrow* in **a**) cationic perfluorocarbon-labeled cells. The corresponding histopathology at day 7 with phase contrast (**e**) and anti-β-gal immunohistochemistry (**f**) demonstrates that implanted cells remain viable and continue to produce the marker enzyme. In (**f**), the *right arrow* indicates cells migrating from the injection site into the brain parenchyma (**d**) MR image of a different animal at 14 days after injection of equal amounts of 4×10^5 neural stem cells in both hemispheres, demonstrating the persistence of ^{19}F signal for 2 weeks. (**g**) Corresponding histopathology showing rhodamin fluorescence from perfluorocarbon-labeled cells, co-localizing with the ^{19}F signal. Size bars are 500 μm (Reproduced, with permission, from Ruiz-Cabello et al. (2008))

In addition, when cells divide rapidly, they dilute the label and become rapidly undetectable (Walczak et al. 2007). These limitations may be resolved by using an MRI reporter gene in a similar fashion, as for instance, the luciferase in bioluminescence imaging or the herpes simplex virus thymidine kinase reporter gene used in positron emission tomography. The development of a reporter gene that is robust and, most importantly, has sufficient sensitivity would be a major breakthrough for the field of MRI cell tracking (Gilad et al. 2007b). So far the use of MRI reporter genes for in vivo cell tracking have been limited. Two different approaches represent the use of the ferritin reporter gene (Fig. 5.6) (Iordanova and Ahrens 2012) and an amide-rich artificial reporter gene that use the proton chemical exchange saturation effect (Gilad et al. 2007a).

5.6 Encapsulation

The last method of labeling cells is by co-encapsulating them in semipermeable alginate capsules (Barnett et al. 2011a). In theory, any contrast agent that is smaller than a molecular weight of about 70 kDa (the pore size of the capsule) can be incorporated. This includes Feridex® (Barnett et al. 2007) and the ^{19}F MRI agents perfluorooctylbromide and polyperfluoroether (Barnett et al. 2011b). The capsules have also been used for labeling MSCs with radiopaque agents, in a rabbit model of hind limb ischemia (Kedziorek et al. 2012).

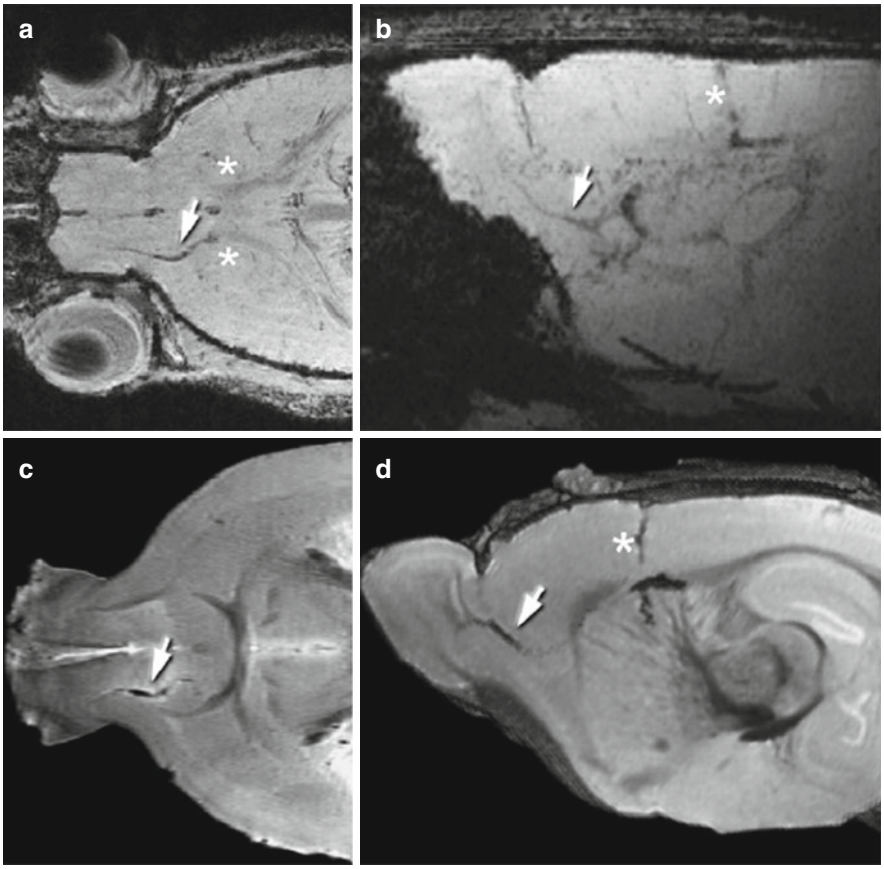

Fig. 5.6 MRI of injected ferritin MRI reporter gene can visualizeendogenous neuroblast migration in neonates. (**a**) Axial projection from several contiguous slices from the T2*-weighted in vivo dataset acquired at 60 μm isotropic resolution 10 days after adenoviral injection in the subventricular zone. *Asterisks* indicate the injection sites. The *arrow* points to a hypointense trail of migrating neuroblasts along the rostral migratory stream (RMS). There is no similar feature on the contralateral side where a control (ferritin-negative) reporter gene was injected. (**b**) Sagittal projection from another mouse imaged in vivo with the same parameters shows the injection site (*asterisk*). An *arrow* shows a hypointense trail of cells in the RMS region. (**c**) Axial projection over the RMS region of an ex vivo 3-D T2*-weighted image acquired with 50 μm isotropic voxels. (**d**) Sagittal 3D projection of another mouse imaged ex vivo with the same parameters. The *asterisk* is the injection site, where the arrow shows a neuroblast stream entering the olfactory bulb (Reproduced, with permission, from Iordanova and Ahrens (2012))

5.7 Summary and Concluding Remarks

MRI cell tracking using SPIO-based contrast agents is a widely used technique, and so far not less than seven clinical trials have been performed (Bulte 2009) including MSCs (Karussis et al. 2010). Cell tracking using paramagnetic agents has been pursued in many preclinical studies, but their potential toxicity and limited sensitivity

make clinical translation unlikely. More recently, ^{19}F MRI has emerged as a promising MRI tracer technique to track cells, and the first perfluorocarbon labels were FDA-approved in 2011 for clinical use. Encapsulation of cells with the addition of contrast agents is an alternative technique that has so far seen limited applications, primarily in pancreatic islet transplantation for treatment of type I diabetes. Finally, MRI reporter genes would in principle be most valuable for MRI cell tracking, but have not yet been widely used and will need to undergo a long regulatory path toward clinical translation. Thus, it is fair to say that SPIO-based cell tracking remains for now the method of choice for clinical studies. Unfortunately, the SPIO formulations previously used clinically are no longer manufactured. The development of a SPIO-based FDA-approved contrast agent optimized for MRI, with superior properties to the less magnetic Ferumoxytol®, is warranted to continue cell tracking in the clinic.

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Strategies for Enhanced, MRI-Guided Targeting of Stem Cells to Stroke Lesions

6

Michael Gorelik and Piotr Walczak

6.1 Introduction

Stroke is currently the second leading cause of death in the Western world (Rymer and Thrutchley 2005) and is a major cause of long-term disability. Patients who suffer ischemic insults have few effective options for therapy. While therapeutic options for the treatment of stroke are being developed on many fronts, evidence of the beneficial effects of stem cell therapy in animal stroke models has grown over the past decade, and several clinical trials have been completed (Kondziolka et al. 2000, 2005). In animal models of stroke, the administration of stem cells has demonstrated a range of diverse, yet modest therapeutic benefits, ranging from cell integration in the brain parenchyma and neuroprotection to modulation of the host immune response (Bliss et al. 2010). However, optimization of stem cell characteristics, timing of delivery, and transplantation method remain essential parameters that must be further improved in order to facilitate the translation of findings in animal studies to clinical trials. Furthermore, utilization of noninvasive imaging techniques that allow for real-time monitoring of stem cell engraftment, distribution, and migration is essential.

Successful cell-based therapy of stroke depends on the efficient delivery of cells to regions of the central nervous system (CNS) pathology. This is particularly true if the goal of stem cell therapy is to replace dying or defunct cells. Intraparenchymal stereotaxic injection is currently the most prevalently used technique for cell delivery to the CNS in animal models (Berman et al. 2011) and in clinical trials (Olanow et al. 2003; Sahni and Kessler 2010). However, intraparenchymal injections are invasive, can lead to nonuniform cell engraftment, and result in limited brain area

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coverage by the graft. Intravenous (IV) systemic cell administration has been proposed as a less invasive delivery route to ensure a broad cell distribution (Janowski et al. 2010). The challenge with this IV approach is achieving specific, high-efficiency binding to target regions in the CNS. Typically, with IV injection, the vast majority of infused cells become trapped in filtering organs (Kraitchman et al. 2005). An alternative method of intravascular delivery is to target the cells to an artery that directly feeds the defunct region. It has been shown that intra-arterial delivery can result in increased cell engraftment to brain lesions (Li et al. 2010) and, notably, with fewer cells found in off-target organs. Furthermore, there is evidence that intra-arterial delivery results in modest functional recovery in animal models of stroke (Shen et al. 2006) and brain trauma (Lu et al. 2001). The focus of this chapter will be to outline the current approaches for enhanced delivery of stem cells to stroke lesions using MRI-guided intra-arterial delivery methods.

6.2 Rationale for Intra-arterial Transplantation in Stroke

Beneficial outcomes of cell-based therapy depend highly on the efficient and safe delivery of cellular therapeutics to brain lesions. Widespread and targeted cell engraftment is particularly important for diseases with multifocal or diffuse pathology, as is characteristically seen in stroke. Such broad engraftment can be achieved by relying either on the extensive migratory potential of cells or on a delivery method that ensures cell placement in the immediate proximity of the affected tissue.

The optimal route of stem cell transplantation for the treatment of stroke is a subject of current investigation. In the vast majority of studies using cell therapy for stroke, the most common modes of transplantation include direct intracerebral injection (IC), as well as intravenous (IV) or intra-arterial (IA) injection. Each has its own limitations, but there is growing evidence that intra-arterial delivery is the most optimal approach for stroke therapy, particularly when combined with techniques that enhance targeted binding of stem cells to stroke lesions.

Direct, stereotaxic brain intraparenchymal injections can be targeted toward the lesion with greater precision, but this is relatively invasive. Stereotaxic methods are efficacious for diseases in which pathology is focal or limited in extent. These methods have been used extensively in animal models (Walczak et al. 2004), as well as in clinical trials for Parkinson's disease (Correia et al. 2005), Huntington disease (Capetian et al. 2009), and ALS (Mazzini et al. 2006). However, while certain cell types have extensive migratory potential (Walczak et al. 2011) with intraparenchymal injection, uniform and complete coverage of larger or multifocal lesions is challenging (Kelly et al. 2004). Such an approach for stroke may require multiple injections to allow the graft to cover the entire area of the stroke lesion, which raises concerns about the safety of the approach. There is also evidence that IC transplantation can lead to poor cell distribution throughout the lesion (Li et al. 2010).

Intravenous (IV) injection is a less invasive approach and an attractive candidate, based on ease of administration and established clinical protocols.

Intravenous approaches to cell delivery have been utilized in animal models and clinical trials (Boncoraglio et al. 2010; Karussis et al. 2010) with reassuring results in terms of safety. However, although intravenous approaches are potentially safer and less invasive than stereotaxic methods, there are, nonetheless, serious concerns about the low efficiency of engraftment (as low as 1–2 %) (Li et al. 2002). With this approach, it is difficult to control cell trafficking, and many reports indicate that transplanted cells filter into off-target organs, such as the lungs or spleen (Fischer et al. 2009; Kraitchman et al. 2005). In addition, given the relative difficulty and expense of acquiring high-quality stem cells for use in clinical trials, IV delivery may prove prohibitive. Finally, recent studies have reported that IV delivery often results in poor cell delivery to the CNS and stroke lesions. Studies report that cells are often trapped in peripheral organs via nonspecific binding, and even in cases in which they bind to the brain endothelium or enter the brain parenchyma, they do so in far fewer numbers compared to intra-arterial delivery (Pendharkar et al. 2010; Walczak et al. 2008). As a result, their therapeutic effect is minimized.

One of the goals of improving intra-arterial delivery of stems cells is to enhance their binding and migratory properties either by selecting unique populations of cells that endogenously express relevant adhesion molecules or by engineering cells to induce that expression. This approach would be counterproductive with IV delivery for cells with such properties. Consequently, IV delivery of highly adherent cells would increase the likelihood of the cells becoming trapped in peripheral organs, thus minimizing therapeutic efficacy and potentially leading to undesired effects.

As clinical trials are being performed in stroke patients (Kondziolka et al. 2000), it is of key importance to design and establish safe and efficient methods to deliver cells to the sites of ischemic brain damage. A major advantage of intra-arterial delivery stems from the potential for widespread engraftment, which is essential for covering larger or multifocal lesions, as well as for high targeting efficiency. Intra-arterial delivery may overcome the limitations of IV delivery by using a more direct route to the central nervous system, such as endovascular selective catheterization of intracerebral vessels for current stroke therapy. This approach is particularly valid for cells that highly express adhesion-docking molecules, ensuring first-pass binding, minimizing cells from reaching off-target tissues, and maximizing the delivery directly to the ischemic lesion, once vessels are reperfused.

Clinical translation of any of these stem cell delivery strategies, particularly IA delivery, will require the use of noninvasive imaging and monitoring techniques. One of the potential disadvantages of IA delivery is that it raises concerns about cells sticking together and creating microemboli. These concerns will necessitate the use of noninvasive, real-time imaging techniques, such as MRI, to monitor and control cell engraftment in order to mitigate potential adverse effects. An IA approach combined with MRI may also open a new area of interventional neuroradiology and provide very precise, minimally invasive delivery of cells to the specific areas of brain pathology in stroke and other CNS diseases in which stem cell therapy is indicated.

6.3 Strategies for Enhanced Targeting of Cells Toward Brain Lesions

Improving intra-arterial delivery methods will necessitate modification of stem cells to promote adhesion to the endothelium and migration toward lesions. Various groups have shown that stem cells have a range of therapeutic benefits, some of which may even be mediated without cells entering the brain (Gutierrez-Fernandez et al. 2011). However, few, if any, studies have convincingly shown this type of benefit for neural or glial progenitors, which are particularly relevant to stem cell therapy in stroke. The therapeutic benefits of neural stem cell transplantation are highly dependent on their homing to the ischemic brain lesions, their appropriate differentiation and/or secretion of growth factors. One obvious requirement and the challenge of using neural stems with an arterial delivery-based approach is to target the cells to the parenchyma of the lesion. Once transplanted cells are infused into arterial blood, they must adhere to activated endothelium near areas of pathology and transmigrate from the blood into the brain parenchyma. This is a complex physiological process that requires several coordinated steps. A useful physiological system to consider is how immune cells migrate toward lesions or areas of inflammation using a variety of surface adhesion molecules. Immune cells reach their destination by sensing chemoattractants, followed by rolling, adhesion, diapedesis, and migration.

One of the most relevant cell adhesion pathways that can be exploited for stem cell targeting is the well-characterized VLA-4/VCAM-1 adhesion pathway utilized by trafficking leukocytes (Engelhardt 2006). The expression of VLA-4 on leukocytes allows them to anchor to the endothelium in the proximity of the brain lesion, respond to chemokine-mediated signaling, cross the blood–brain barrier, and migrate to areas of immunopathology. While the VLA-4/VCAM-1 adhesion pathway has been shown to be critical, there are myriad other adhesion receptors and molecules involved in this process, some of which are discussed below. Furthermore, there is coordination between the molecules expressed on activated endothelium and the migrating cell.

The ability of neural stem cells to adequately reach their target lesions after intra-arterial delivery likely depends on a similar process. However, this is not the normal physiological role of neural stem cells, and their expression of the full range of adhesion molecules required for such targeting is an area of current investigation. Enhancing the ability of stem cells to reach their targets is critical for improving intra-arterial delivery methods. It is known that, following ischemic insult in rodents, cell adhesion molecules, including ICAM-1, ICAM-2, and VCAM-1, are upregulated between 24 and 48 h, and there are other subsets of adhesion molecules that change their expression pattern as the lesion develops (Justicia et al. 2006). Thus, an understanding of the types of molecules expressed on the endothelium near the area of the stroke lesion is important for selection of the cells used for therapy, which must have the corresponding adhesion receptors to enable migration into the brain parenchyma. An understanding of how these molecules change throughout the progression of a stroke lesion will aid in integrating information about the optimal timing of stem cell transplantation with the underlying biology of the brain endothelium in stroke.

There are two approaches toward enhancing targeted delivery of stem cells. One method relies on selecting and enriching cells that endogenously express surface adhesion receptors, which are critical for the process of adhesion and transmigration. The other approach relies on engineering cells to express the appropriate adhesion receptors. Each approach has benefits and drawbacks that must be assessed for future clinical applications.

6.4 Engineering Cells for Enhanced Targeting

Stem cells, including neural, glial, mesenchymal, and umbilical cord cells, have been shown to have modest therapeutic benefits in animal models of stroke using a range of transplantation methods. Even for intravenous and intra-arterial delivery routes, a range of cell types have been used, and there is currently no consensus as to which cell type has the greatest potential. Indeed, it is likely that a range of cell types may be appropriate, depending on the type of pathology and the molecular targets of the therapy. It would be optimal to have the flexibility to combine the desired cell type and intra-arterial targeting for a maximum therapeutic effect. Potential problems related to this strategy are that the cells of choice may not express the required molecules for endothelial adhesion. Although this is a new area of investigation, most cell types appropriate for stroke transplantation, particularly those of neural lineages, do not express the full complement of adhesion receptors utilized by migrating immune cells. Thus, while a particular cell type may be therapeutic for stroke, it may not be suitable for intra-arterial delivery. Engineering such cell types by overexpressing the appropriate adhesion molecules presents a path forward for enhancing targeted, intra-arterial delivery of cells that do not endogenously express the required adhesion receptor.

This approach was used in a recent study that sought to enhance binding of glial-restricted precursor cells (hGRP) to brain endothelium following intra-arterial delivery by expression of VLA-4 (Gorelik et al. 2012). VLA-4 was shown to be absent in hGRPs, and its transient expression was achieved using DNA plasmid transfection. In order to promote binding and adhesion, recipient animals were injected with lipopolysaccharide (LPS) to mediate global brain endothelial activation, as measured by the expression of VCAM-1. Animals underwent surgery to secure access to the internal carotid artery (ICA) and ligation of all ICA extracerebral branches. VLA-4 expressing human glial precursors was found to bind extensively to the cerebral endothelium in animals that received LPS, as measured by MRI and immunohistological analysis. Animals that received naïve hGRPs showed drastically reduced binding, even in those animals that received LPS injections (Fig. 6.1). This demonstrates both the importance of adhesion molecule expression on transplanted cells, as well as the necessity of endothelial activation.

This proof-of-principle study demonstrated that the modification of stem cells with adhesion molecules significantly increases specific first-pass binding to activated brain endothelium. However, it has some notable limitations. Although engineering of cells with molecules relevant for inflammatory brain lesions does lead to

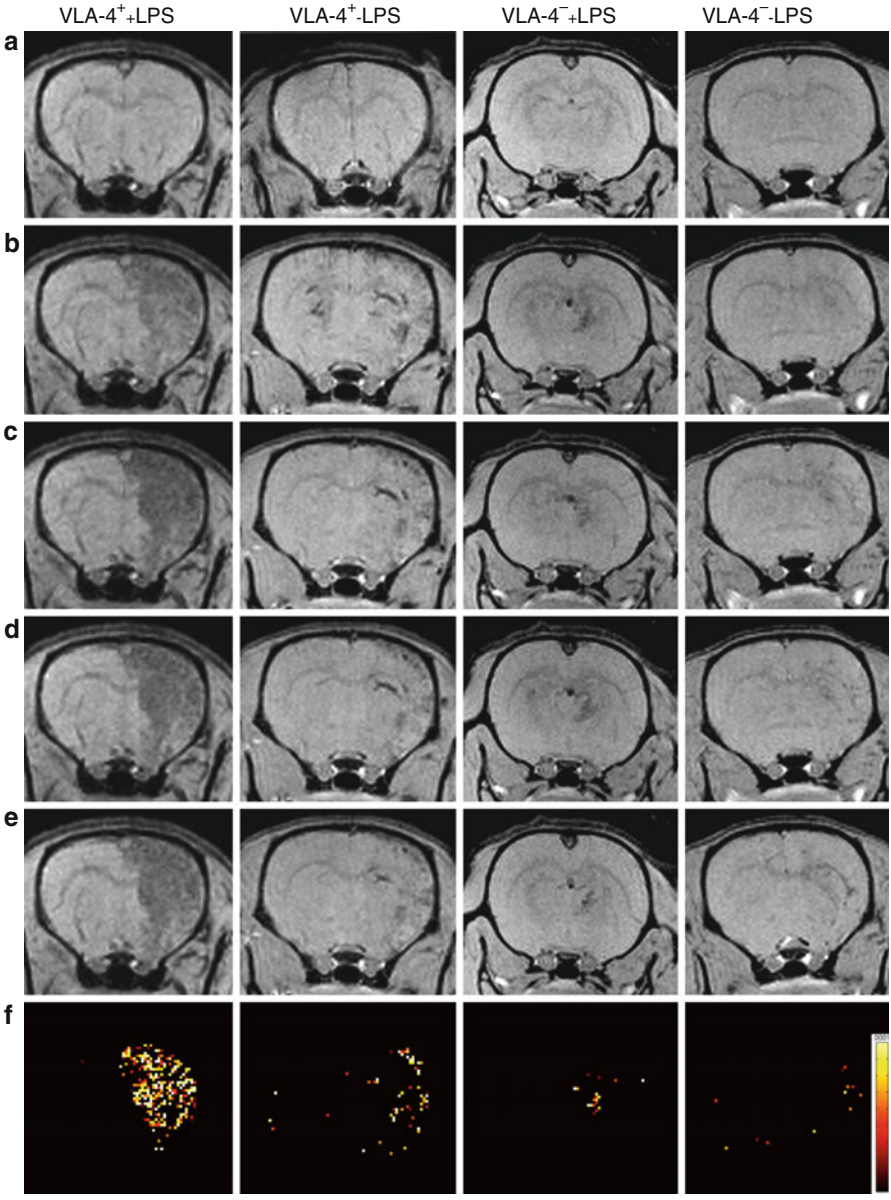

Fig. 6.1 In vivo real-time quantitative MRI of cerebral docking of cells following intra-arterial delivery. Representative T2*-weighted brain images acquired before injection (**a**), after the first injection of 1×10^6 cells (**b**), 10 min later after the second cell injection of 1×10^6 cells (**c**), 20 min later (**d**), and the final image obtained 30 min after the first cell injection (**e**). Brain regions with hypointense signals indicate docking of cells to the cerebral endothelium. Pixel-by-pixel analysis of pre-injection MR images (**a**) vs. MR images at $t = 30$ min (**e**) with a significance threshold of $p < 0.001$ (**f**). A higher number of hypointense pixels can be found for the VLA-4+ cells following LPS treatment (Adapted with permission from Gorelik et al. (2012))

endothelial capture, it still has to be established whether the cells are able to extravasate to the brain parenchyma. The process of diapedesis is complex and requires many steps beyond just adhesion. In this study, only the early postgrafting period was evaluated, with the vast majority of the cells localizing within the cerebral vasculature, as demonstrated by immunohistochemistry.

The purpose of the study was to examine short-term binding, but this raises several questions, including whether or not the expression of VLA-4 is sufficient to mediate migration into the brain parenchyma. While stroke lesions have been shown to upregulate VCAM-1 at lesion sites, this study used a global model of inflammation in which VCAM-1 was upregulated throughout the brain. Further research is needed to determine whether the homing of cells to cerebral ischemia can be mediated by just VLA-4, or in concert with other molecules, but also how the evolution of stroke over time affects intra-arterial targeting.

Another area for exploration is methods by which stem cells are modified to enhance their adhesive and migratory properties. In the study described above, DNA plasmid transfection was used with the rationale that there would be a robust but temporary increase in the expression of VLA-4 sufficient for binding. Presumably, once cells bound to activated brain endothelium, they would use other molecules to enter the brain while the levels of VLA-4 would decrease. Other methods of engineering cells to express non-endogenous proteins include lentiviral transduction (Poeschla et al. 1998), mRNA transfection (Van Tendeloo et al. 2001), or even direct delivery of peptides to the cells (Robbins et al. 2001). The impact of introducing new proteins into cells must be examined closely, as non-endogenous proteins may influence the physiology of the cell.

6.5 Enriching Stem Cells for Endogenous Adhesion Molecules

Engineering cells with adhesion molecules is an approach that requires manipulation of cells, and it has yet to be determined whether and to what extent such manipulations will affect the capacity of the cells to differentiate and mediate their therapeutic effects in vivo. An alternative approach for enhancing stem cell targeting toward stroke lesions is to enrich for cells that endogenously express the relevant adhesion molecules. Although neural stem cells do not express the same complement of adhesion molecules as immune cells, several groups have shown that certain neural stem cells do indeed express important adhesion receptors that are used endogenously for migration during neural development. For example, neural stem cells, similar to leukocytes, express the chemokine receptors CCR1, CCR2, CCR5, CXCR3, and CXCR4 (Tran et al. 2004). These cell types can be enriched by various methods, such as fluorescence-assisted cell sorting (FACS), i.e., selecting a pure population of cells that express the desired adhesion molecule.

A study by Guzman et al. demonstrated that CD49d, the α4 subunit of VLA-4, is expressed on a subpopulation of neural stem cells, and FACS sorting for this antigen can significantly increase homing to the ischemic brain tissue following intra-arterial

delivery (Guzman et al. 2008). In this study, hypoxia–ischemia was induced in mice and intracarotid cell injection was performed 48 h after stroke. The study found that 2 weeks after intracarotid injection in stroked animals, NSCs were detected in the perivascular space of capillaries and within larger vessels, similar to what was observed in Gorelik et al. (2012). Furthermore, significant numbers of cells were found in the brain parenchyma ipsilateral to the hemisphere of the stroke. This suggests that these cells underwent transendothelial migration into the injured brain parenchyma. There was also good correlation between markers of the activated endothelium, such as VCAM-1 and lectin, and the distribution of the injected stem cells (Fig. 6.2). One question this study leaves open is the role of other adhesion proteins or molecules that may have been involved in promoting entry into the brain parenchyma.

In a subsequent study, Guzman et al. (Andres et al. 2011) investigated the role of the CCL2/CCR2 adhesion pathway to determine its role in the enhanced targeting of therapeutic homing of intra-arterially delivered NSCs. CCL2 is a chemokine recognized by its receptor, CCR2. In this study, NSCs isolated from CCR2+/+ and CCR2−/− were delivered intra-arterially into wild-type mice and CCL2−/− mice using the hypoxia–ischemia stroke model described above. Three days after transplantation, NSCs were detected predominantly in the ischemic area of the cortex and striatum, with greater numbers of cells found in animals that received CCR2+/+ vs. CCR2−/− grafts. Furthermore, a comparison of animals that received CCR2+/+ vs. CCR2−/− grafts at 14 days revealed significantly more cells in the area of the stroke in animals that received CCR2+/+ vs. CCR2−/− grafts. An important observation noted by the authors was that regardless of whether animals received CCR2+/+ vs. CCR2−/− grafts, many of the cells in the brain were positive for the neuronal markers, NeuN and TuJ1, and the astrocyte marker, GFAP (Fig. 6.3).

These studies demonstrate the feasibility of flow cytometry as a method of enriching NSCs for adhesion markers that are critical for enhanced targeting using intra-arterial delivery. They also demonstrate that cells selected for these markers were able to survive, enter the brain parenchyma from the vasculature, migrate toward stroke lesions, and mediate a modest therapeutic effect. The benefits of this approach stem from the relative simplicity of FACS as a technique by which it purifies cell populations without genetic manipulation. These studies also identified several molecules that appear to be important for intra-arterial targeting. As in Gorelik et al. (2012), the VLA-4 adhesion receptor was shown to be critical, and cells selected for CD49d were able to enter the brain.

However, in both of these studies, only neural stem cells were used. In each case, it was necessary to purify cells to enrich for the desired markers. In the Guzman studies, 25 and 77.1 % of cells expressed CD49d and CCR2 prior to sorting, respectively. It is unclear what percentage of cells would coexpress both markers. Thus, for highly specific targeting, it remains to be seen how difficult it would be to sort for all the critical markers needed to mediate effective targeting toward stroke lesions. Furthermore, it is unclear whether other cell types that are relevant for stroke therapy, such as oligodendrocyte precursors, endogenously express similar

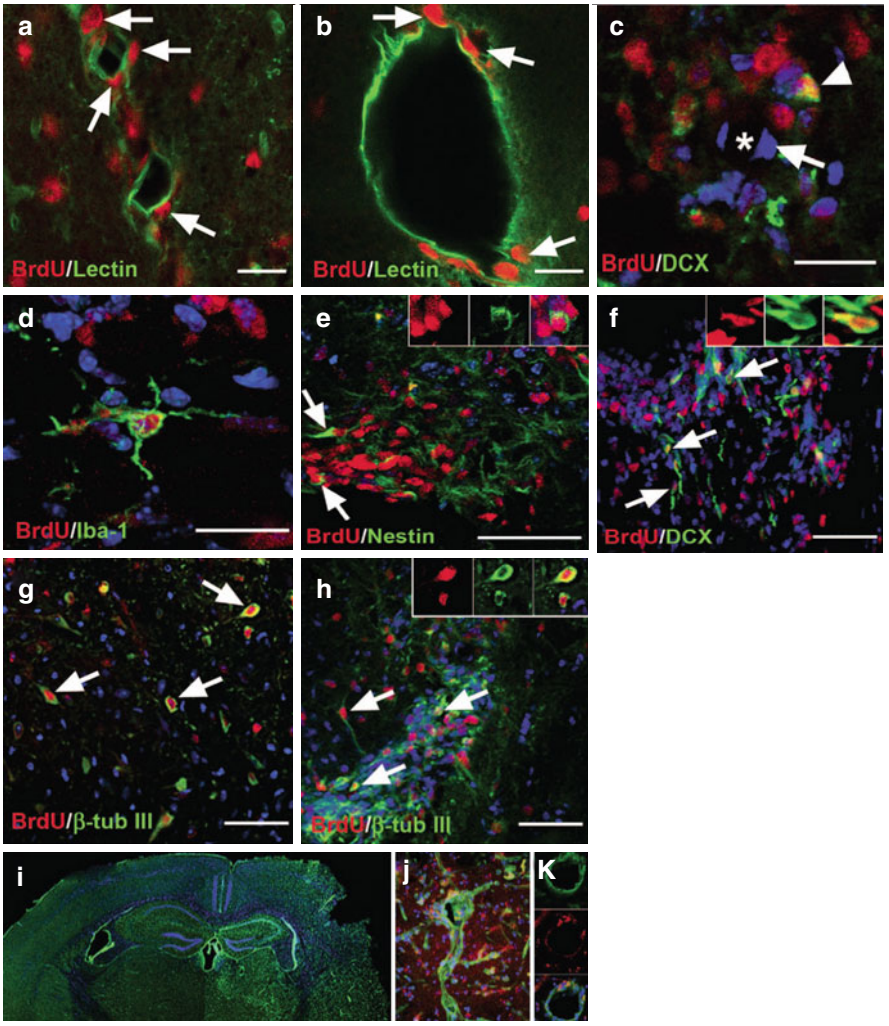

Fig. 6.2 Location and phenotype of NSCs after intra-arterial injection into the mouse brain after stroke. (**a**) BrdU-prelabeled NSCs (*arrows*) were found in close association with capillaries (BrdU in *red* and lectin in *green*) and larger blood vessels (**b**) in the stroked hemisphere (scale bar 20 μm). (**c**) High magnification of a capillary cross section (*asterisk* in the vessel lumen, endothelial cell, *arrow*). BrdU (*red*) and doublecortin (*green*) double-labeled cell in the perivascular niche (*arrowhead*). (**d**) Fifteen percent of the BrdU (*red*)-positive cells coexpressed the panmonocytic marker Iba-1 (*green*), representing NSCs that had been phagocytosed (**a**–**d** scale 20 μm). (**e**) Immature NSCs (BrdU in *red*), positive for nestin (*green*, *arrows*), were found in the subventricular zone. (**f**) NSCs (BrdU in *red*), with the morphology of migrating neuroblasts (*arrows*) and positive for DCX (*green*), were found in the stroke border zone. (**g**) Numerous NSCs expressed b-tubulin III (*green*) in the striatum and the cortex (**h**) adjacent to the stroke (*Inset* in **e**, **f**, **h** high magnification of double-positive cells in *red and green* channels and overlay; **e**–**h** scale bar, 50 μm). Two days after stroke, there was a high expression of lectin (**i**) and VCAM-1 (**j**–**k**) in the stroked hemisphere (Adapted with permission from Guzman et al. (2008))

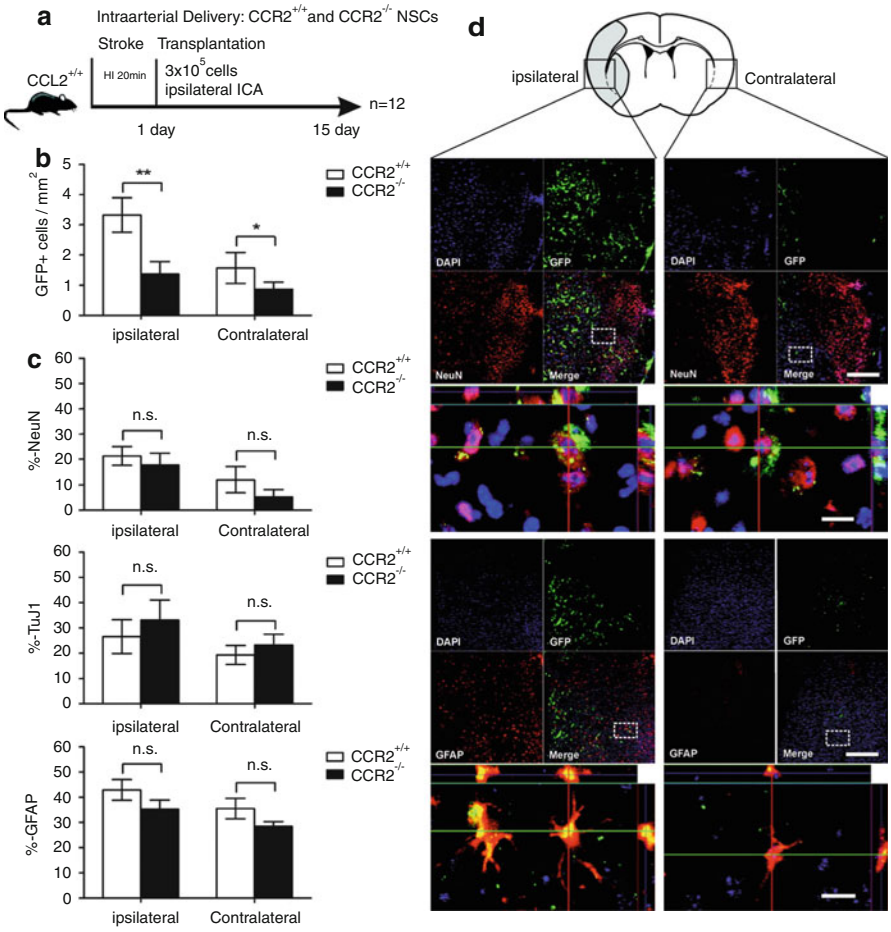

Fig. 6.3 Number of recruited cells and differentiation of intra-arterially delivered neural stem cells (NSC) in the postischemic brain at 14 days after transplantation. Experimental setup (**a**). Significantly higher numbers of cells were found in the ipsilateral hemisphere in the CCR2+/+ group (**b**). Colocalization studies with the neuronal (NeuN, neuronal class III b-tubulin (TuJ1)) and astrocytic (GFAP) markers did not show any significant differences in NSC differentiation in vivo between the CCR2+/+ group and the CCR2−/− group (**c**). Representative confocal photomicrographs from the ipsilateral and contralateral hemispheres demonstrating colocalization of NeuN (*red*, *upper panel*) and GFAP (*red*, *lower panel*) with green fluorescent protein (GFP)-positive (*green*) NSC (**d**). Data are mean ± standard error of the mean ($n = 12$ per group, two independent experiments). $^{*}P < 0.05$, $^{**}P < 0.01$. *n.s.* indicates not significant. Scale bars are 200 μm (*split color panel*) and 20 μm (high-magnification orthogonal images) (Adapted with permission from Andres et al. (2011))

types of markers that appear critical in mediating transendothelial migration and targeting to stroke lesions. The hGRPs used in Gorelik et al. did not endogenously express VLA-4 and could not be considered for FACS enrichment. Thus, this approach may be limited to only specific cell sources.

6.6 Real-Time MRI Monitoring of Cell Engraftment

One obstacle that has hampered the advancement of stem cell transplantation for stroke is inadequate methodology that would allow stem cell characterization and real-time monitoring of cell fate following transplantation into living organisms. Several techniques for noninvasive cellular imaging have been developed, including intravital multiphoton microscopy (Tran-Dinh et al. 2006), bioluminescence (Tang et al. 2003), PET (Adonai et al. 2002), and MRI (Walczak et al. 2008). However, MRI appears to be the best-suited and most clinically applicable technique for the intravascular delivery of stem cells for stroke.

A successful method of stem cell delivery to stroke lesions must be balanced between the efficiency of targeting and, most importantly, the safety of the procedure. While increased cell adhesion to the cerebral endothelium is highly desirable for intra-arterial infusion, this approach may potentially amplify the risk of the formation of microembolisms (Li et al. 2010; Walczak et al. 2008) and compromise essential cerebral blood flow, both of which are critical to avoid in stroke patients. To ensure the safety of this approach, it is critical to monitor cell delivery noninvasively in real time. The use of MR imaging with superparamagnetic iron oxide (SPIO) nanoparticle-labeled cells, which have been used clinically (Karussis et al. 2010), allows for real-time visualization of both the accuracy of cell delivery (Barnett et al. 2007; Karmarkar et al. 2004) and the relative amount of cell engraftment (Walczak et al. 2008).

A study by Walczak et al. investigated the feasibility of monitoring intra-arterial delivery of mesenchymal stem cells, after transient ischemia, with laser Doppler flowmetry (LDF) measurements of cerebral blood flow in conjunction with MRI cell tracking (Walczak et al. 2008). In vivo T2*-weighted MRI detected the engraftment of (SPIO) labeled cells within the right internal carotid artery ICA vascular territory, as represented by the presence of strong signal voids, and also confirmed with high-resolution ex vivo MRI (Fig. 6.4). The engraftment of injected MSCs was found to occur primarily throughout the ipsilateral hemisphere, indicating that cells engrafted during the first pass—without entering the systemic circulation. After 1 day, cell infusion cells were located within the cerebral capillary bed, and, after 10 days, some cells were found to have entered the parenchyma of the cerebral cortex.

In addition to demonstrating the feasibility of real-time monitoring of engraftment, the results of this study also highlight some critical safety issues for intra-arterial delivery.

The study found that the delivery of MSCs into the carotid artery resulted in a high variability of cell engraftment. In some animals, there was no change in cerebral blood flow, as measured by laser Doppler flowmetry (LDF), with no cells detectable in the brain by MRI or histology, and, in other animals, the LDF signal drop was moderate with moderate cell engraftment. The authors also report that, in some recipients, the injection caused a significant (80–90 %) reduction in LDF and the rapid death of the animal within 2–4 h. This finding highlights the importance of using real-time monitoring to ensure the safety of intra-arterial delivery. For

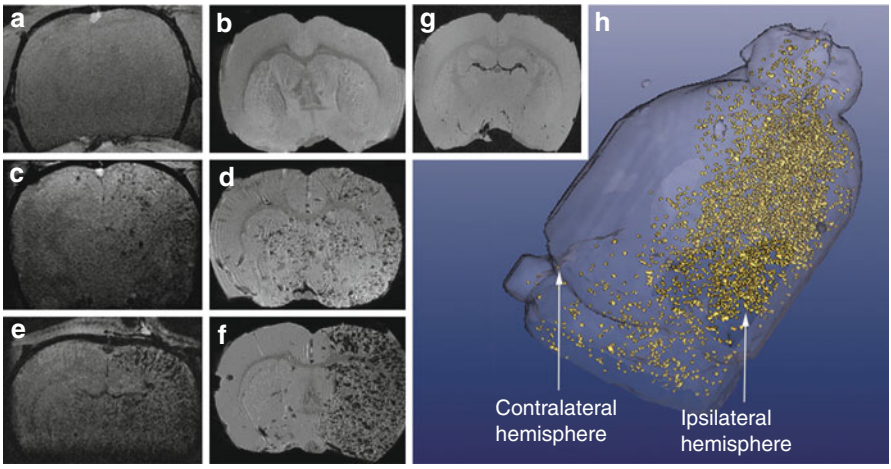

Fig. 6.4 MR images of intracerebral cell engraftment after IA (**a**–**f**, **h**) and IV (**g**) injection. MSCs appear as hypointense spots on T2*-weighted images. A high variability of cell engraftment has been observed, ranging from practically no engraftment, shown in (**a**) in vivo and (**b**) ex vivo MRI, moderate engraftment (**c**, **d**), and extensive engraftment (**e**, **f**). No engraftment could be detected after IV injection (**g**). (**h**) 3D reconstruction of cellular distribution in the brain of the example shown in (**e** and **f**). Cells distributed widely throughout the right brain hemisphere, with a prevalence for the area directly fed by the right internal carotid artery; the territory of the anterior and posterior cerebral arteries also exhibited significant engraftment. Very few cells were detected in the contralateral (*left*) hemisphere, suggesting that cells did not circulate systemically before arriving at their final location (Adapted with permission from Walczak et al. (2008))

example, given the potential variability of engraftment, the rate of infusion could be adjusted in real time or stopped altogether if cerebral blood flow were decreased.

The ability to track the magnetically labeled cells in vivo using MRI can provide longitudinal information regarding migration, homing potential, cell distribution, and an approximation of the amount of grafted cells present in the tissue. These parameters are important, as they are closely related to the therapeutic outcome following transplantation (Li et al. 2010). A study by Li et al. used MRI to monitor these various parameters following intra-arterial, intravenous, and intracerebral transplantation of magnetically labeled NPCs in a model of ischemic brain injury. The cell administration route remarkably affected the final pattern of cellular distribution, even though the various groups had a similar level of ischemic injury. MRI revealed that transplanted NPCs engrafted more diffusely and in small clusters throughout the ipsilateral brain, whereas the typical cellular distribution pattern after IC and IV administration was characterized by larger cell clusters, with numerous cells accumulating within the lesion boundary. These results indicate that IA delivery results in earlier engraftment, more uniform and widespread distribution, and a larger number of transplanted cells in the host brain than IC or IV delivery. However, although IA administration showed encouraging results, it was also accompanied by higher rates of mortality during cell delivery, consistent with the findings of Walczak et al. The cause of the mortality was likely due to impeded cerebral blood flow.

In addition to monitoring engraftment, real-time MRI allows visualization of the bio-distribution and migration of transplanted cells after IA or IV delivery. This is critical to the development of intravascular stem cell therapy for stroke, as histological methods of analysis are inadequate for gaining a complete understanding of the distribution of cell engraftment and cannot account for the dynamics of initial and secondary redistribution (Pendharkar et al. 2010). A study by Pendharkar et al. combined bioluminescent imaging (BLI) and MRI to monitor cell engraftment using IV or IA delivery (Pendharkar et al. 2010). Using BLI, the study found significantly higher photon counts from head ROIs in the IA groups compared to the IV group, indicating cerebral engraftment. Conversely, IV-transplanted animals demonstrated a significantly higher signal in the torso region compared to IA-injected animals, suggesting that the majority of IV-injected cells distribute to the lungs. MRI corroborated the increased engraftment in IA vs. IV transplantation.

6.7 Imaging Techniques to Ensure Safety of Intra-arterial Delivery

A crucial safety parameter for clinical and experimental use of intra-arterial cell delivery techniques is the evaluation of tissue perfusion. As mentioned above, intra-arterial delivery introduces the risk of microembolism formation, thus compromising cerebral blood flow. The application of noninvasive imaging techniques may address this problem by monitoring brain tissue perfusion, oxygenation, and physiology during or soon after transplantation.

MRI, with a breadth of contrast mechanisms available, is well-suited for measuring brain hemodynamic parameters. There are several established MRI-based techniques that work well, with an early identification of an ischemic penumbra—an area of hypoperfused tissue that is viable but at risk of infarction (Hossmann 1994). The identification of an ischemic penumbra and subsequent thrombolysis treatment increases survival and reduces disability, especially with early intervention. Diffusion-weighted MRI (DWI) (Sotak 2002)and perfusion-weighted MRI (PWI) (Minematsu et al. 1992) can detect regions of cellular membrane depolarization and cerebral blood flow (CBF) reduction, respectively, and the PWI–DWI mismatch visualizes the maximum area of abnormal hemodynamic supply. Other MRI techniques to assess cerebral microcirculation are dynamic susceptibility contrast-enhanced (DSC) MRI and steady state susceptibility contrast-enhanced MRI (Dijkhuizen and Nicolay 2003). These techniques measure signal changes after the intravascular injection of an exogenous contrast agent. Arterial spin labeling (ASL) is a technique that exploits endogenous arterial water as a perfusion tracer. ASL methods are now advanced enough to provide routine, quantitative sequences for brain ASL (Alsop 2012), and several ASL techniques have been used in the study of acute stroke (Detre et al. 1999). However, the main limitation of ASL is the relatively low SNR, which may preclude imaging with a resolution sufficient enough to detect local changes in cerebral blood flow following stem cell injection. An emerging MRI technique, called pH-weighted imaging (pHWI), may better predict the

boundaries of the ischemic penumbra and areas with compromised blood perfusion (Sun et al. 2007).

Other techniques that may be relevant to monitoring hemodynamic parameters in the context of stem cell injection include laser Doppler flowmetry (LDF) (Dirnagl et al. 1989), near infrared spectroscopy (NIRS) (Villringer and Chance 1997), and CT perfusion imaging (Wintermark et al. 2006). LDF is routinely used for the non-invasive measurement of microcirculation and is widely used to evaluate stroke surgery, as well as the treatment of cerebral ischemia in animal models (Dirnagl et al. 1989). CT perfusion (CTP) is a functional imaging technique that provides important information about the capillary-level hemodynamics of the brain tissue. CTP is critical in determining the extent of irreversibly infarcted brain tissue (infarct "core") and the penumbra. This is achieved by generating parametric maps of cerebral blood flow, cerebral blood volume, and mean transit time (Konstas et al. 2009). Perfusion CT has been used for the early detection of ischemic stroke (Koenig et al. 1998) and appears to be suitable for the early detection of cerebral ischemia while also providing valuable information about the extent of perfusion disturbances.

Conclusion

Several recent experimental studies have highlighted the enormous potential of stem cell transplantation as a promising therapy for stroke. The therapeutic benefits of stem cells range from integration into the host circuitry and a reduction of host cell death to the induction of host brain plasticity, trophic support, and the modulation of inflammation. In many of these cases, the therapeutic benefits provided by stem cell grafts depend on transplanted cells entering the brain parenchyma near the areas of pathology. The challenge for stem cell therapy in stroke is providing a safe and efficient method for targeting stem cells to stroke lesions. MR-guided intra-arterial delivery, combined with the utilization of stem cells enriched or engineered for properties that enhance specific targeting, is a promising approach for the treatment of stroke. MRI provides real-time, noninvasive monitoring during transplantation and also reveals information about the bio-distribution of transplanted cells. The use of cells enhanced for lesion targeting increases the efficiency of cell engraftment and also enables the selection of the most relevant adhesion molecules that will aid in migration toward stroke lesion sites. These techniques for cell targeting and adhesion require the monitoring of brain perfusion during cell injection to ensure safety of this delivery method and to ensure that stem cell therapy for stroke is a realistic and obtainable goal.

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Image-Guided Injection and Noninvasive Monitoring of Tissue Regeneration in the Stroke-Damaged Brain

7

Chung-Hsing Chou, Francesca Nicholls, and Michel Modo

7.1 Introduction

Ischemic stroke is the result of a blockage of blood supply to a part of the brain. This induces a cascade of physiological events that leads to cellular death of all cells not receiving oxygen and nutrients (Lipton 1999). However, the affected area (infarct and peri-infarct) consists of a heterogeneous pattern of damage (del Zoppo et al. 2011). At the core of infarction, where there was no blood flow and no collateral blood supply, all cells rapidly die (Ledezma et al. 2009). Even in the absence of reperfusion, this cell death invokes an invasion of macrophages that clear cellular debris, but there is also an erosion of extracellular matrix (ECM) leading to a cavitation (Clark et al. 1993). Upon reperfusion of this area, blood vessels might be severely damaged, and small bleeds might ensue, potentially causing even further damage to the neuropil. However, given a complete loss of blood supply, as well as time, this tissue is irrevocably lost (Baron 1999).

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In the area surrounding the core, the so-called peri-infarct area, the impact of the occlusion is less severe due to collateral blood supply or some residual vessel perfusion. Nevertheless, there is cellular as well as tissue damage in the peri-infarct area, although it is less clear-cut (del Zoppo et al. 2011). Some cells undergo apoptosis, microglia are activated, and astrocytes react by dedifferentiation and proliferation to produce a glial scarring. Acutely, this area is also known as the penumbra, as some cells within this area can be saved by providing neuroprotective factors or by reducing the metabolic demand of the tissue by cooling the subject (i.e., hypothermia) (Baron 1999). Nevertheless, if no intervention is introduced, a large proportion of this tissue will gradually die and contribute to the behavioral impairments that ensue after stroke. Unfortunately, none of these neuroprotective agents have been efficacious in clinical trials (Hoyte et al. 2004).

Behavioral impairments are due to cellular loss (especially neurons) but are also due to damage to axonal tracts that pass through or close to the site of infarction. These deficits develop rapidly as normal cellular function can no longer be sustained. Their phenomenology is dependent on the region(s) that is affected by the stroke and therefore can be very diverse. The most prominent impairments are associated with the core of damage, and these often remain permanently impaired. In the case of a stroke, it is therefore important to remove the cause of blockage as quickly as possible (Lopez et al. 2011). At present, the only effective pharmacological agent for stroke is recombinant tissue plasminogen activator (rt-PA), which can reduce the extent of damage. Dysfunctions associated with the peri-infarct area, however, can also recover as those cells that survived the infarction can take over some functions. This plasticity can also occur in more remote connected areas, so-called diaschisis (Mountz 2007). Plasticity of the remaining brain is therefore an important aspect in "spontaneous recovery" (Johansson 2011).

Repopulating this peri-infarct area with new brain cells is potentially an attractive approach to promote some behavioral recovery (Burns and Steinberg 2011). Neural stem cells during fetal development produce the brain cells in the neuropil (i.e., neurons, astrocytes, oligodendrocytes). In contrast, endothelial cells forming blood vessels and microglia providing immune surveillance are peripheral cells that gradually invade the central nervous system but are nevertheless required to maintain brain tissue. Importantly, these cells also produce ECM that is providing the structural support to maintain cells and compartmentalize tissue (neurovascular unit versus neuropil versus white matter).

In some cases, neural stem cells migrate to the peri-infarct area, even from remote injection sites (Modo et al. 2002). However, there is also evidence that human neural stem cells do not readily migrate, at least in rodent brains, and therefore mainly exert their effects close to their site of injection (Smith et al. 2011). The site of injection therefore becomes an essential aspect to ensure efficacy. Behavioral recovery due to implantation of neural stem cells can be the consequence of cellular replacement (neurons, astrocytes, and/or oligodendrocytes) but can also be the product of the secretion of growth factors from these cells that modulate brain plasticity (Burns and Steinberg 2011). Implanted cells can also affect blood vessels or the immune system which in turn can affect neuronal functions. The microenvironment (lesion core, penumbral tissue or peri-infarct area, or normal tissue) within which cells are

implanted therefore is likely to influence the function of these cells, but the effects of the cells might also differ. As behavioral impairments are due to specific functions being supported by different regions affected by stroke, it is also reasonable to assume that depending on which affected regions cells are implanted will influence which type of deficits are likely to improve after injection. Placement of cells into an appropriate microenvironment and locale are crucial parameters that ensure the efficacy of cell therapy. To warrant a correct injection, it is essential that 3-dimensional imaging techniques, such as magnetic resonance imaging (MRI) or computed tomography (CT), provide noninvasive visualization of the stroke-damaged brain that can be used to derive target coordinates.

7.2 Image-Guided Injection of Stem Cells and Biomaterials

Foremost of all, noninvasive images need to define criteria to select subjects for inclusion in an efficacy study (Yoo et al. 2011). Although this is common practice in clinical trials, preclinical studies rarely use imaging-based criteria. Selection criteria can more clearly define a homogenous group and highlight particular neurobiological features thought to be relevant to efficacy (Heiss 2011). For instance, inclusion and exclusion criteria for an efficacy study could define a minimum hyperintense area on T_2-weighted MR images. Very small lesions (e.g., <10 mm^3 in the rat) revert to an isointense contrast over 14 days and hence should be excluded if lesion volume is one of the main outcome measures. Therefore, it is important to have a robust imaging paradigm setup to detect the evolution of the infarct (Helpern et al. 1993; Knight et al. 1994). Diffusion-weighted MRI, however, affords a more reliable detection of tissue changes in smaller lesions. As deficits are a function of the size of the lesion, as well as how many anatomical structures are affected by the infarction, the efficacy of implanted cells is likely to be influenced by these factors. For instance, an injection of human neural stem cells into the peri-infarct area was more efficacious in animals with purely striatal lesions compared to those that also exhibited cortical damage (Smith et al. 2011).

Preimplantation imaging therefore is an important component in surgical planning. In human patients, many of these aspects have been developed for stereotactic neurosurgery, in cases of tissue resection/biopsy or deep brain stimulation (Risholm et al. 2011). For this, high-resolution structural images are required to delineate the neuroanatomy and provide a reference to plan a burr hole on the skull as well as the trajectory of surgical access and the needle (Bible et al. 2009a). However, care must be taken to minimize iatrogenic complications that could lead to new dysfunctions. Structural images by themselves might therefore be insufficient. Additional details, such as the presence of major blood vessels, white matter, or essential functional activity will ideally be mapped onto the neuroanatomical images (Kekhia et al. 2011). Pinching through a major vessel could lead to a hemorrhage that would cause significantly more damage and severely compromise a patient with an ischemic stroke. Cutting through white matter could also lead to some axonal connections being disrupted and cause additional deficits. Although needle trajectories to deep-seated brain structures will always have to traverse overlying brain regions, it is

important to consider the functionality of these regions. For instance, prodding through speech areas would severely compromise a patient, especially since the speech center is lateralized. Readjusting the trajectory to go through an adjacent motor area that is bilaterally represented can minimize the potential functional impact of the surgery (Signorelli et al. 2003).

In preclinical studies, implantation sites are commonly predefined based on stereotactic coordinates from a neuroanatomical atlas. These coordinates are applied to all animals irrespective of their lesion size or location. Image-guided surgical planning is rarely applied. Although the biological impact of the trajectory is similar in terms of the damage caused by the needle, behavioral testing is generally too insensitive to detect any functional consequences of cutting through axons in the corpus callosum or damage to the motor cortex. Nevertheless, placement of cells is an important aspect to ensure that implanted cells encounter the same microenvironment as well as being located in an appropriate position in relation to the lesion (Bible et al. 2009a). Although animal studies are much more homogenous in terms of damage than human patients, important variations in lesion territory exist. It is therefore important to use this information for surgical planning and establish conditions under which cells can be most efficacious. For instance, if damage is restricted to striatal tissues, striatal neural stem cells placed into the striatum are likely to be more efficacious than cells placed in the cortex (Smith et al. 2011). To repair cortical damage, neural stem cells derived from the cortex might be required to provide some positional specification (e.g., PAX6) and region-specific differentiation. If the infarct affects the striatum and cortex, it is conceivable that implantations in striatum and cortex are required to deliver an optimal outcome. Imaging is required to define the lesion territory and to define coordinates that will place cells into an appropriate locale (Fig. 7.1).

However, tissue characteristics will influence the fate of implanted cells. For instance, injection of cells into striatal tissue with a normal cytoarchitecture is unlikely to support the long-term survival of these cells, and tract damage invokes glial scarring that can adversely affect this functional tissue. In contrast, injecting cells in the peri-infarct area is a different scenario. Here, a large proportion of neuronal cells have been lost, and injection of cells will not cause as much disruption to the tissue. The diffusion of the injectate encounters fewer cellular and tissue barriers. Moreover, there is an efficient blood supply in this area as well as an ECM that can support novel cells. However, discriminating these two types of tissue on T_2-weighted images would be extremely challenging (Baron 1999; del Zoppo et al. 2011). Imaging techniques that indicate metabolic activity, such as ^{18}Fludeoxyglucose Positron Emission Tomography (FDG-PET), or that describe tissue characteristics, such as diffusion-weighted MRI or blood oxygen level dependent (BOLD) contrast, are more apposite (Baron 1999; Holmes et al. 2012). However, at present, no detailed investigation has been conducted to establish if subdivisions within the peri-infarct area can be reliably distinguished from the lesion cavity and normal tissue surrounding the area of damage. Further development of noninvasive imaging is therefore warranted to provide an apposite image-guided injection of cells.

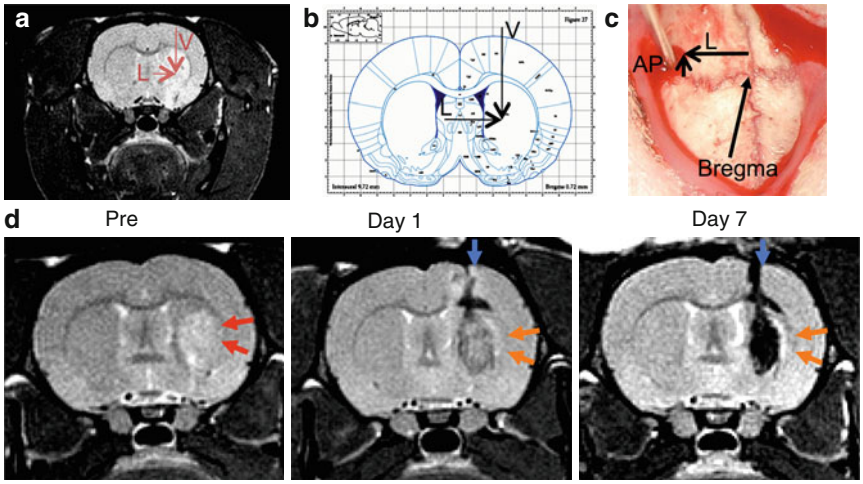

Fig. 7.1 Image-guided injection. T_2-weighted MR images reveal the stroke cavity as a hyperintensity (**a**) (*AP* anterior-posterior, *L* lateral *V* ventral). Based on these images, it is possible to match this slice with the corresponding anatomical landmarks in a stereotactic atlas (**b**). Measurements of coordinates in relation to its slice, in terms of ventral and lateral coordinates in relation to bregma, allowing an appropriate drilling of a burr hole (**c**) and injection of cells into the center of the lesion cavity. This approach allows the image-guidance of injections directly into the lesion cavity and its monitoring over time, as shown here for neuroscaffolds consisting of neural stem cells on PLGA microparticles (**d**) (stroke lesion (*red arrow*), injection tract (*blue arrow*), injection site of PLGA particles (*orange arrow*))

The lesion cavity is readily identified on T_2- as well as diffusion-weighted MRI scans (Knight et al. 1994). However, implantation into the cavity results in cells invading host tissue but does not result in tissue regeneration. At present, it remains unclear if cells injected directly into the lesion cavity will promote behavioral recovery as no studies have reported yet an image-guided injection. It is nevertheless clear that cells by themselves do not regenerate tissue inside the lesion cavity. It is generally assumed that the lesion cavity is void of cells and tissue, but it is conceivable that major blood vessels, such as the middle cerebral artery, could survive after reperfusion. In contrast, capillaries will vanish with the clearance of tissue debris. The lesion cavity forming from the core of the ischemic infarct is therefore generally void of cells and ECM and mostly just contains extracellular fluid.

Restoration of this lost tissue therefore poses a major challenge but if possible could provide a new neural substrate capable of supporting behavioral functions (Dihne et al. 2011). To achieve the establishment of new tissue, however, a multitude of favorable conditions have to be engineered. Foremost of all, there is a need to provide a structural support for cells to integrate into. Scaffolding microparticles to which cells can attach or an ECM bioscaffold can achieve these conditions and retain implanted cells within the lesion cavity (Bible et al. 2009b, 2012; Zhong et al. 2010). Image-guidance of injections is essential to achieve this. Lesion volumes are rather large (20–100 mm^3), and sufficient biomaterial, as well as cells, needs to be

injected in the fluid-filled cyst to provide sufficient material to create a de novo tissue. Injection of similar volumes would create a very significant damage in host tissue. Therefore, injection volumes and locations need to be established from preimplantation images to ensure the safety of this procedure but also to provide evidence of the newly established tissue.

7.3 Noninvasive Monitoring of Tissue Regeneration

In situ tissue engineering is a further development of cell therapy (Yu and Morshead 2011). To generate new tissue, not only will implanted cells need to remain and survive within the lesion cavity, but there is also a requirement for a vascular network and site-appropriate differentiation as well as integration with the host brain tissue. Growth factors can be secreted from biomaterials to guide these processes (Lam et al. 2010). However, the number of processes that need to be controlled is staggering and is a dynamic process. Similar processes are well described in both mammalian development and invertebrate regeneration (Tanaka and Ferretti 2009). However, the unique situation of mammalian brain regeneration will require an equally unique set of processes to ensure that an appropriate tissue forms. Although some processes will be similar to those seen in development (Cramer and Chopp 2000), it is likely that they will be orchestrated in a different way, with sequence alteration and some entirely different processes. Moreover, by implanting cells and providing the structural substrate for these cells, these processes invoked might even be very different from those involved in true tissue regeneration as occurs in invertebrates.

To gain control over the processes and to ensure they synchronize to form a tissue, it will be important to have appropriate noninvasive-imaging techniques that can monitor these processes over time. Some biomaterials will have relaxivity properties and hence can easily be detected using MRI, but this inherent contrast will make it difficult to interrogate what changes occur within the lesion territory (Bible et al. 2009b). Therefore, a tunable or induced contrast in biomaterials might be desirable. Incorporating contrast agents into biomaterials, such as hydrogel, can make these visible on MRI without interfering with structural T_2-weighted images (Karfeld-Sulzer et al. 2011; Kim et al. 2012). The release of contrast agents from biomaterials in conjunction with the release of growth factors also potentially provides a means to monitor their distribution and release kinetics in vivo (Onuki et al. 2010). This of course will be essential to also understand how factors released from biomaterials interact with the host brain. Multifunctional and multimodal materials (Yim et al. 2011) might therefore be needed to allow the simultaneous monitoring of various processes.

Detection of biomaterials used to engineer tissue, however, should not interfere with the visualization of tissue formation (Fig. 7.2). Only T_2-weighted MR images will be insufficient to demonstrate tissue formation (Bible et al. 2012). Conventional MR contrast agents affect ^{1}H and therefore are likely to affect T_2-weighted images, whereas novel classes of contrast agents, such as ^{19}F or chemical exchange saturation transfer (CEST), are likely to be better candidates to report on the presence of biomaterials. However, for regenerative imaging, it is even more important to be

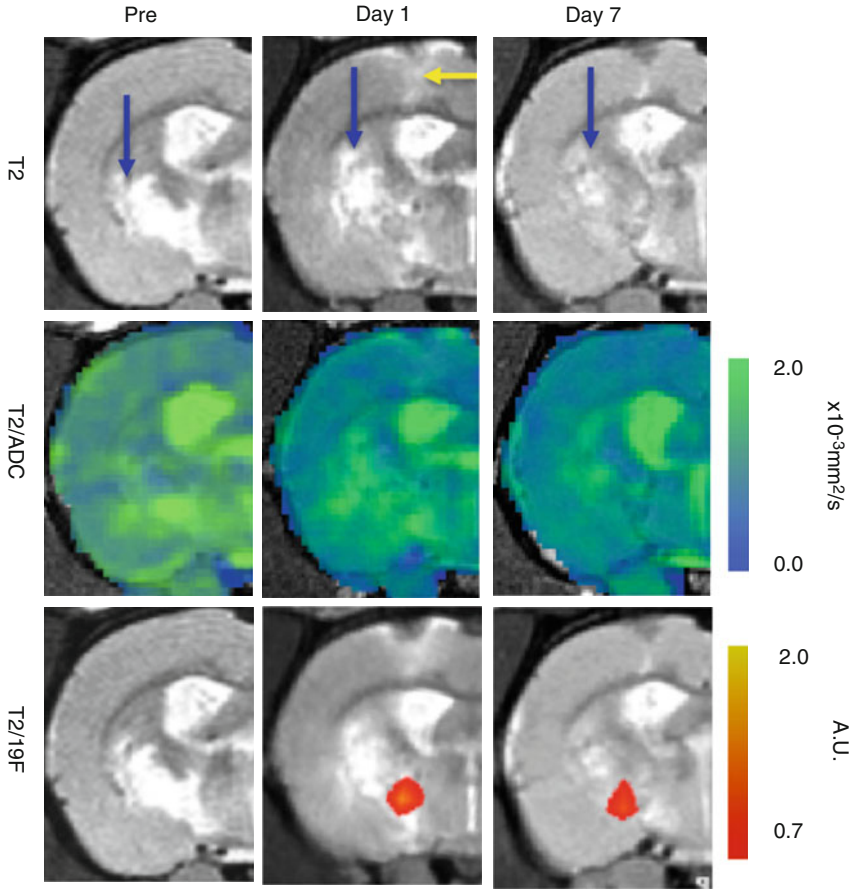

Fig. 7.2 Monitoring in situ tissue engineering. As middle cerebral artery occlusion results in quite heterogeneous damage and in some even sham occlusion producing neurological signs, it is essential that a preimplantation noninvasive image demonstrates that a lesion was present prior to the injection of the cell/biomaterial mix. It is here evident that there is a lesion cavity (*blue arrow*) prior to our injection of human neural stem cells (labeled with a ^{19}F MRI contrast agent) with an ECM bioscaffold. On day 1, based on the ^{19}F MRI, it is evident that cells were indeed injected into the lesion cavity, but the lesion environment is still largely a liquid that is hyperintense on the T_2-weighted image and very few barriers for diffusion are evident. However, on day 7, the T2-signal almost returned to a normal signal, but there was still a significant diffusion deficit in the area of infarction. It is also evident that cells are still present within this environment. The injection of cells/biomaterials also creates some minor damage in the cortex (*yellow arrow*). This approach therefore provides unequivocal evidence of changes in the lesion cavity due to the injection of biomaterials and cells but at present does not yet comprehensively describe the processes involved in tissue formation

able to visualize biological processes rather than the biomaterials. One of the most basic elements of this is to monitor the presence of transplanted cells in vivo. Various strategies and imaging paradigms have been described for this cellular imaging (Modo et al. 2005). MRI has the advantage of high spatial resolution in vivo. In the

context of regenerative imaging, contrast agents, such as ^{19}F, are more desirable, as they can report on the distribution of a large number of cells without interfering with lesion detection. However, these exogenous labeling strategies will always suffer from potential leakage of agents from cells and reuptake by host cells. Moreover, exogenous tagging is unlikely to report on the live/dead status of implanted cells and will be diluted by cell division. Reporter gene strategies therefore might be an alternative, but currently for MRI, these mainly focus on the ^{1}H signal (Gilad et al. 2008). Alternatively, bioluminescence (BLI) can detect live cells, and the reporter gene will be propagated even during proliferation (Daadi et al. 2009). However, BLI suffers from a low spatial resolution, and it is doubtful if this approach could be applied to human patients. Therefore, significant work remains in developing an appropriate strategy to detect live implanted cells noninvasively.

An additional challenge is to indicate if implanted cells have generated an appropriate type of tissue. Establishing if a tissue is indeed developing is significantly more challenging that merely establishing if implanted cells have differentiated into one appropriate neuronal phenotype. Foremost of all is the question if a tissue has developed with appropriate diffusion barriers, as these are essential to keep the tissue together. Using diffusion-weighted MRI, the establishment of these diffusion barriers (e.g., ECM, axons) can be ascertained (Bible et al. 2012). Diffusion barriers nevertheless also limit the flow of extracellular fluid typically found inside the lesion cavity. Hence, with the establishment of diffusion barriers, it is important to deliver nutrients and oxygen to the de novo tissue by angiogenesis as well as arteriogenesis. Monitoring the emergence of an appropriate blood supply to this area is consequently crucial for its long-term survival. Perfusion MRI can demonstrate appropriate flow of blood to the area, whereas MR angiography can visualize the presence of major blood vessels (Seevinck et al. 2010). However, all these aspects are also present in tumors, and therefore, the site-appropriateness of the de novo tissue also requires interrogation.

In the absence of existing tissue, there are no cues to instruct site-appropriate differentiation of implanted cells. These cues need to be delivered through biomaterials or predifferentiation of cells (El-Akabawy et al. 2011). The molecular signature of the tissue can potentially be monitored noninvasively using magnetic resonance spectroscopy (MRS) (Loewenbruck et al. 2011; Manganas et al. 2007). MRS can establish a particular metabolite profile for a given tissue, such as striatum or cortex, as these metabolites are dependent on the variety of elements contained within the tissue (Brownell et al. 2004), and they thereby provide a very general means to establish what aspects of the appropriate tissue have been regenerated (Ross et al. 1999). For instance, MRS of the rat striatum clearly indicates the presence of GABA and glutamate (Roffman et al. 2000), two key neurotransmitters in this neuroanatomical structure. Therefore, any replacement tissue needs to demonstrate that similar levels of neurotransmitters can be generated. Additionally, MRS can report on the health of the tissue with lactate being upregulated in dying cells (Woo et al. 2010) and phosphocholine being upregulated in proliferating cells (Gillies et al. 1994). Future studies have to nevertheless demonstrate that MRS can indeed provide a reliable discrimination between these different processes.

Despite potentially replacing the lost striatal tissue with a new tissue that has the biochemical properties of the original tissue, this does not necessarily translate into recovery due to an integration of cells within neural networks of the host brain. Glial scarring and the lack of appropriate axonal connections can potentially form a tissue that is not connected. Therefore, appropriate in vivo methods need to be developed to establish if the newly formed tissue is responsive to input from the host brain and vice versa that the regrown striatum can indeed elicit activity in the host brain. Pharmacological MRI is one means to investigate if the new striatum is specifically responsive to agents that stimulate striatal neuron activity (Chen et al. 1999; Roberts et al. 2007). After fetal tissue transplants, functional MRI has, for instance, also been used to demonstrate the integration of the new tissue with host activity (Bluml et al. 1999). It is also conceivable that if a new tissue integrated with the brain, it will fire spontaneously and in the absence of a regulatory feedback can be the focal point for epileptic discharges. Electroencephalography (EEG) can help to establish if inappropriate discharges are indeed occurring, in the absence of behavioral manifestations. Appropriate monitoring of the functional integration of cells will hence also be important to ensure the safety of this approach.

Although the connections between new and existing tissue is thought to be essential to provide an integration into networks that produce behavior, merely establishing physical synaptic connections is unlikely to be sufficient to ensure that this new tissue is indeed functionally integrated (Hicks et al. 2007). During development, these new neurons are likely to be a "blank slate," and they will require functional activity from other cells to establish and maintain a functional integration (Dobrossy et al. 2010). Rehabilitative training specifically geared toward integrating these newly established cells and connections is likely to be a major requirement to ensure the efficacy of this approach. It is likely that the rehabilitative needs of this tissue are very different from those currently employed to promote recovery after stroke. Rehabilitation after stroke is geared toward promoting plasticity in remaining brain regions but is not necessarily appropriate to train a new tissue or guarantee its integration with other brain regions. Therefore, significant challenges remain ahead.

Conclusion

Invertebrates can regrow complex anatomical structures, such as a limb, composed of multiple types of tissue, as well as completely repopulate, for instance, dopaminergic cells in the substantia nigra (Berg et al. 2011). However, it is unclear if they can actually regenerate brain tissue. In mammals, no regeneration of brain tissue occurs, despite local neurogenesis. We have demonstrated that by using biomaterial as a scaffolding support, it is possible to retain cells within a lesion cavity and at least generate a primitive type of tissue that contains cells, but lacks, for instance, a vasculature (Bible et al. 2009b, 2012). Unfortunately, this is but a very small step in a long journey. We have here outlined a variety of different challenges that lie ahead as well as the need for appropriate noninvasive imaging required to serially monitor these processes. It is only by engaging these issues in a gradual fashion that we will gain a better understanding of what aspects are going to be essential to create de novo tissue that can impact behavioral recovery.

It is important to be cognizant that we might encounter novel issues that we cannot currently solve, but there might also be insurmountable obstacles, and it is likely that we might never achieve behavioral recovery. Still, as bioengineering and our understanding of the brain's connectivity are rapidly developing, there is reason to be cautiously optimistic that we might be able to achieve in situ tissue engineering inside the stroke-damaged brain. Considering the impact of stroke and the potential to affect long-term disability by regenerating/replacing brain tissue, this is an effort we cannot shy from.

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Tracking of Autologous VSOP-Labeled Mesenchymal Stem Cells in the Sheep Brain Using 3.0 T MRI

8

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8.1 Introduction

Ischemic stroke is among the pathological conditions with highest mortality and morbidity in Western societies (Langhorne et al. 2011). Despite significant improvements of clinical treatment regimen, particularly the introduction of the stroke unit concept, the disease is still one of the major causes of death and the most important reason for permanent disability in adulthood (Bonita et al. 2004).

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Treatment interventions mainly focus on the recanalization of the occluded vessel with alteplase (recombinant tissue plasminogen activator, tPA) as the sole effective and FDA-approved therapeutic approach (Falluji et al. 2012). Next to the thrombolytic approach, the use of anticoagulative/antiplatelet drugs for the prevention of secondary ischemic events (Emre et al. 2007) and decompressive hemicraniectomy as a symptomatic therapy in case of malignant middle cerebral artery (MCA) occlusion (Vahedi et al. 2007) are implemented in clinical stroke management guidelines. Even though the therapeutic time window for thrombolysis by tPA was recently extended from 3.0 to 4.5 h upon stroke onset (Lansberg et al. 2009), numerous contraindications as well as an increasing risk for hemorrhagic transformation at later time points (Shobha et al. 2011) still exclude most patients from treatment. Interventional approaches aiming at clot removal or local thrombolysis can be safe and effective within a time window of up to 8.0 h (Natarajan et al. 2009) but require an experienced team of interventional radiologists and are restricted to a few, highly specialized centers.

Hence, there is a strong demand for alternative therapeutic approaches with stem cell and cell-based therapies currently being among the most promising options (Burns and Steinberg 2011). Evidence for neuronal differentiation and/or beneficial effects after transplantation were shown for a multitude of stem and progenitor cell populations in experimental studies using animal models of focal cerebral ischemia. These comprehensive investigations revealed the therapeutic potential of embryonic stem cells (Nagai et al. 2010), neural progenitor cells (Minnerup et al. 2011), and induced pluripotent stem cells (Jensen et al. 2011). However, those cell populations are either available from non-autologous sources only or bear the risk of malignant transformation after transplantation (Seminatore et al. 2010). This currently limits the applicability of the cells in a clinical setup. Autologous sources of adult stem cells may represent a reasonable alternative. Bone marrow-derived stromal cells (BMSC), a kind of mesenchymal stem cells (MSC), were reported to reduce functional deficits (Shen et al. 2007b) and ischemic lesion size (Ukai et al. 2007) and do not show signs of malignant transformation and excessive tissue overgrowth after transplantation. Notably, these cells may enhance angiogenesis and neurogenesis following stroke (Bao et al. 2011) and are further supposed to modulate reactive gliosis in a pro-regenerative manner (Shen et al. 2008). Therapeutic effects were also reported for MSC from other sources including adipose tissue (Kranz et al. 2010) and umbilical cord matrix (Lin et al. 2011), corroborating the anticipated therapeutic potential of MSC. Consequently, the safety of cell-based therapies using autologous bone marrow-derived MSC was already assessed in early-stage clinical trials (Bang et al. 2005; Honmou et al. 2011). However, relevant modes of action as well as the optimal transplantation route still need to be elucidated. Detailed knowledge about the biodistribution of cells after local or systemic administration will complement our understanding of the therapeutic mechanisms and is considered important for assessing safety aspects, which is also essential from a regulatory perspective.

8.1.1 Approaches for Stem Cell Labeling and Tracking in Large Animal Models

Different labeling techniques are applied to track administered cells in vivo. Bioluminescence imaging (BLI) gives relevant spatial and semiquantitative information on cell distribution and homing (Jang et al. 2010) but is restricted to smaller organisms due to the limited light penetration through biological tissue. Single photon emission computed tomography (SPECT) uses gamma-emitting marker radioisotopes and allows reliable signal quantification in experimental animals and human patients but provides poor spatial resolution and anatomical information. SPECT was already applied to detect technetium-99 m-labeled bone marrow-derived cells in patients after intra-arterial delivery (Barbosa da Fonseca et al. 2010), even though repeated investigations have to be considered with care to minimize the radioactive burden. Magnetic resonance imaging (MRI) was shown to be feasible for cell tracking in both, experimental subjects (Muja and Bulte 2009) and human patients (de Vries et al. 2005), and MRI scans can be performed repeatedly and without relevant risks, enabling time course studies. Moreover, excellent spatial and anatomical information can be obtained due to the submillimeter resolution of this imaging modality. In experimental setups, T2- and T2*-weighted imaging sequences are best suitable for tracking locally (Kim et al. 2008; Daadi et al. 2009) or systemically (Stroh et al. 2006) administered cells in rodent models of stroke after paramagnetic cell labeling. Small bore 7.0 T, 9.4 T, or even 17.6 T dedicated animal scanners were used for this purpose. Application of those high field strengths and tailored scanning sequences allows for isotropic resolutions of less than 100 μm, with excellent sensitivity toward labeled cells, detecting 100 or less cells in the brain (Stroh et al. 2005). However, the use of high field strength (7.0 T or more) human scanners is mainly restricted to experimental studies at the moment. These scanners will not be available in most centers in the foreseeable future.

International expert committees recommend the validation of cell-based therapeutic strategies for stroke in large animal models before translating experimental approaches into clinical trials. Better anatomical discrimination between gray and white matter in the gyrencephalic brain as well as the assessment of specific cell delivery options in large animal models are considered advantageous (Savitz et al. 2011). Moreover, autologous cell therapies may be performed more easily as compared to small animal species. The use of large animal models is also relevant from an imaging perspective, since the investigation of both human patients and large animals necessitates the use of clinical MRI scanners with lower field strengths (commonly 3.0 T), wider bores, and different resonance coil sets. Larger bore diameters result in magnetic field inhomogeneities which have to be compensated and taken into consideration when translating experimental imaging sequences to clinical scanners. These aspects can be investigated best by using a combination of large animal models and clinical scanners. The ovine brain has been reported feasible for advanced imaging studies using clinical scanners, and a model of focal cerebral ischemia is available in the species (Boltze et al. 2008).

A comprehensive set of experimental studies was designed to reveal the optimal labeling protocol as well as the in vitro detection limits for ovine MSC in using T2* sequences at 3.0 T. The in vivo detection limit for autologous ovine MSC and their discrimination from hemorrhagic events in the sheep brain were assessed after local cell administration using the same magnetic field strength, while methods for the histological identification of transplanted cells have also been investigated. Finally, pilot experiments for cell tracking after intravenous MSC delivery in an ovine stroke model have been performed. The most notable results and consequences for translational approaches will be discussed in this chapter.

8.2 MRI of VSOP-Labeled Cells In Vitro

Bone marrow cell samples were harvested and processed as described elsewhere (Dreyer et al. 2012) from anesthetized healthy adult Merino sheep. Ovine MSC were separated from MNC according to a widely used separation method (Pittenger 2008). Very small superparamagnetic iron oxide particles (VSOP) were used for MSC labeling. It has been previously reported that incorporation of VSOP into cells causes a significant signal loss in T2*-weighted sequences (Arbab et al. 2003). Cytotoxic effects of VSOP labeling in lower concentrations (up to 3.0 mM) have not been observed in spleen- (Stroh et al. 2006) and bone marrow-derived MNC, as well as human cord blood MNC (Stroh et al. 2009). Only transient oxidative stress was reported to be induced in rodent macrophages at 3.0 mM (Stroh et al. 2004). The development of tailored labeling protocols is recommended to assess potential detrimental effects of labeling on the particular cell population used (Stroh et al. 2009). Thus, the previously reported VSOP labeling protocols were transferred to and optimized for ovine MSC, primarily by assessing the effect of different VSOP labeling molarities.

The transverse relaxation times of 9×10^5 cells in 3 mL PBS, either labeled with 1.5 or 3.0 mM VSOP, were measured at 0.47 T/20 MHz using a Bruker Minispec relaxometer and compared to relaxation time of unlabeled cells (Fig. 8.1a). There was a significant shortening of the relaxation time from 1,888 ± 171 ms (100 %) to 583 ± 190 ms (31 ± 11 %) for 1.5 mM ($p < 0.001$) and 434 ± 146 ms (23 ± 8 %) for 3.0 mM ($p < 0.001$). Moreover, incubation with 3.0 mM VSOP significantly reduced relaxation time as compared to 1.5 mM incubation concentration ($p < 0.05$).

Next, the impact of VSOP cell labeling on MSC viability was assessed. Cell numbers were counted using the trypan blue exclusion method (Fig. 8.1b) after the different labeling conditions. As compared to controls (100 %), there was a drop in the number of vital cells to 90 ± 19 % for 1.5 mM ($p > 0.05$) and 86 ± 16 % for 3.0 mM ($p < 0.05$) immediately after labeling. Viability rates remained almost constant until 4 h after VSOP incubation (91 ± 18 % for 1.5 mM and 89 ± 14 % for 3.0 mM). Additional MTT testing did not reveal signs of relatively reduced or enhanced metabolic activity between control conditions (set at 1.0) and cell samples immediately after labeling (1.5 mM: 1.10 ± 0.09; 3.0 mM: 0.98 ± 0.14;

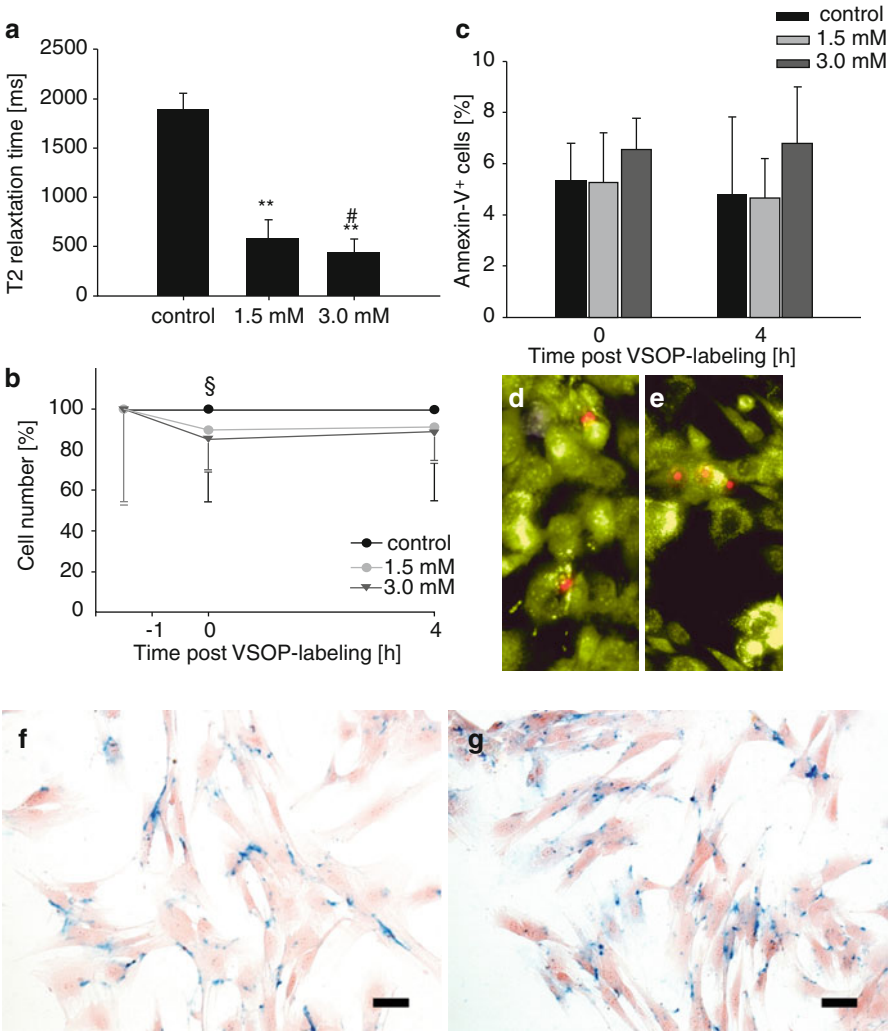

Fig. 8.1 VSOP-labeling of ovine MSC. Incubation of MSC with VSOP led to a significant reduction of T2 relaxation time (**a**, $p<0.001$). Increasing VSOP concentration from 1.5 to 3.0 mM further significantly shortened T2 relaxation time (**a**, $p<0.05$). Cell labeling slightly reduced viable cell numbers. This difference was statistically significant only immediately after labeling with 3.0 mM (**b**). Apoptosis rates, measured by annexin-V-staining, were not significantly affected by VSOP incubation at both molarities (**c**). This finding is in accordance with results from a life/dead-cell assay using acridine-orange (vital cells, *green*) and ethidium-bromide (dead cells, *orange-red*; **d**, **e**). Prussian-Blue-staining clearly showed intracellular iron deposits after labeling with 1.5 mM (**f**) and 3.0 mM (**g**). $^{**}p<0.001$ (versus control); #/§$p<0.05$ (versus 1.5 mM/3.0 mM). Scale bars: 50 µm

$p=0.154$) and 4 h later (1.5 mM: 0.90 ± 0.10; 3.0 mM: 1.00 ± 0.10; $p=0.940$). Moreover, apoptosis rates, being measured by cytometry-based detection of annexin V staining in MSC, were comparable to control conditions 4 h after

labeling (Fig. 8.1c) with apoptosis rates being 4.79 ± 3.04 % in control MSC populations versus 4.55 ± 1.57 % (1.5 mM) and 6.80 ± 2.20 % (3.0 mM) in labeled MSC, respectively ($p > 0.05$). Similar results were obtained using a live/dead assessment by acridine-orange (vital cells, green)/ethidium-bromide (dead cells, orange-red) staining (1.5 mM: Fig. 8.1d; 3.0 mM: Fig. 8.1e). Thus, it may be concluded that there is a loss of viable cells during VSOP labeling, especially after labeling with 3.0 mM VSOP. However, this cell loss is comparatively small and only occurs directly after labeling. Differences between 1.5 and 3.0 mM incubation molarities in other viability assays, even though not statistically significant, underline the necessity of additional long-term investigations. A follow-up of at least 24 h following labeling is required to detect a possible late detrimental effect of VSOP labeling on ovine MSC at 3.0 mM higher incubation molarities and to predict possible consequences for further research. Exemplary samples of labeled MSC after Prussian blue (PB) staining are given in Fig. 8.1f (1.5 mM) and Fig. 8.1g (3.0 mM). Since labeling with 3.0 mM VSOP gave superior result regarding relaxation time shortening, this concentration was chosen for MSC labeling in all further experiments.

In order to prepare experiments for MRI-based tracking of autologous MSC in vivo, the detection limit of the cells was assessed in gel phantoms. Those gel phantoms – in contrast to conventional small-scale test tube samples – can closely resemble cell distributions observed upon local in vivo administration or after homing of systemically administered cells to a particular site. Gel phantoms mimic both the magnetic properties of brain parenchyma and the diffusion constants. Thus, gel phantoms with injected cells may serve as ideal model for tailoring imaging sequences (Stroh et al. 2005) and are commonly used to investigate detection limits of labeled cells.

Since a potential homing of VSOP-labeled cells to an ischemic lesion in the brain can be supposed to result in areal signal extinctions in zones bordering the central infarct after systemic administration (Stroh et al. 2006; Tsai et al. 2011), agarose gel phantoms were used to simulate this effect with MSC being embedded in thin layers within the phantoms. Phantom one contained 0 unlabeled and 100, 500, and 5,000 labeled MSC, respectively, while phantom two contained 100,000 unlabeled and 1,000; 10,000; and 100,000 labeled cells (Fig. 8.2a).

Gel phantoms were placed in a Siemens Trio 3.0 T clinical MRI scanner equipped with an 8-channel coil. This coil is suitable for examinations of both humans and large animals and was therefore used in the experiments. A T2*-weighted sequence was conducted according to Table 8.1. Detection limits were assessed by three researchers with experience in radiological assessments but blinded to the experimental conditions. These researchers had to decide whether or not signal losses, presumably evoked by VSOP-labeled cells, are visible in a certain layer. Only in case all three investigators independently decided that this was the case, a cell number was declared detectable under the particular condition.

In the T2* sequence, gel phantoms were visualized as bright, homogenous structures with very rare signs for embedded air bubbles. No hypointensities based on signal losses were observed in layers containing no cells (Fig. 8.2b) or 100,000

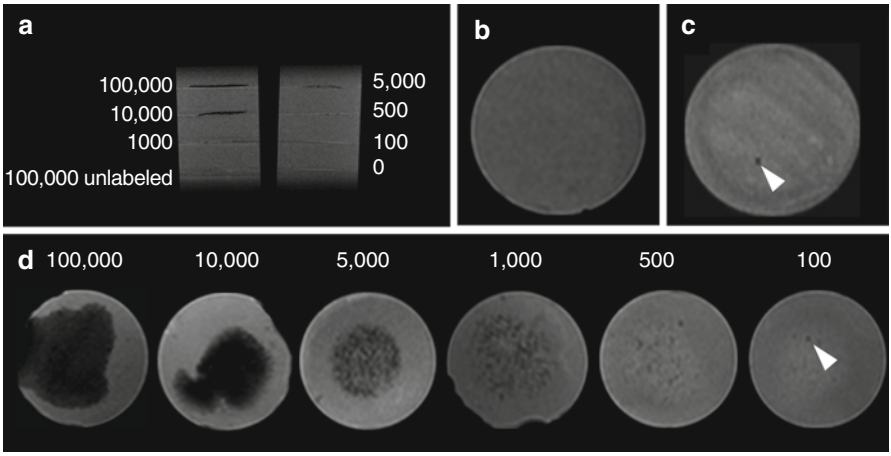

Fig. 8.2 Detection limit of VSOP-labeled MSC in gel phantoms at 3.0 T. Agarose gel phantoms containing horizontal layers of 0–100,000 labeled MSC or 100,000 unlabeled MSC were manufactured and measured at 3.0 T by applying a T2* sequence (see Table 8.1) (**a**). Cell-free gel layers (**b**) and 100,000 unlabeled MSC (**c**). Air bubbles inside the gel layers were rarely observed, causing small, punctual signal extinctions (**c**, *white arrow head*). Planar signal voids were observed in layers containing 1,000 or more cells, whereas 500 cells caused punctual signal losses within the layer (**d**). 100 cells (**d**, *white arrow head*) were not unambiguously discriminable from scattered air bubbles (**c**), thus the detection limit was found at 500 cells at 3.0 T in this experiment

Table 8.1 Design of T2* MR sequences for in vitro and in vivo investigations

	Sequence	Coil	TR/TE	Flip angle (°)	Bandwidth (Hz)	Averages	Voxel size (mm)	Scan time (min)
In vitro	T2* FLASH 3D	8 channel coil	620/20	20	200	1	0.55×0.47×0.6	314
In vivo	T2* FLASH 3D	4 channel flex coil	620/20	20	200	1	0.83×0.66×0.5	127

unlabeled MSC (Fig. 8.2c, note small air bubble, white arrow head) in horizontal slices. In contrast, 100,000 labeled cells became clearly visible (Fig. 8.2d) as planar signal losses which were clearly discriminable from the gel. Similar results were observed for 10,000; 5,000; and 1,000 cells with declining sizes of the planar signal losses (Fig. 8.2d). Five hundred labeled MSC per layer (Fig. 8.2d) could also be detected, but signal losses appeared as small punctae and not as planar signal extinctions. 100 cells per layer were not unambiguously detectable as distinction between the punctual signal extinction and rarely occurring single air bubbles was not possible (Fig. 8.2d, white arrow head). Thus, the detection limit was defined to range at 500 cells at 3.0 T, superior to 1.5 T (5,000 cells, data not shown).

8.3 MRI of VSOP-Labeled Cells In Vivo

In the next step the detection limit of VSOP-labeled cells in the ovine brain was investigated. This is of particular importance since the presence of blood vessels or hemorrhages in the brain can lead to false-positive results. Hemorrhages can be of traumatic origin (damage to small intracerebral vessels during local cell injections) or may occur consecutively after major territorial ischemia.

However, scans of defined duration (more than 5 h) can only be performed with difficulties in sheep, since prolonged anesthesia (>4 to 8 h) may have detrimental metabolic effects and can cause hypothermia with compensatory shivering, which severely affects image quality. Very long anesthesia (8 h or more) will result in poor recovery rates in sheep (Boltze et al. 2008). Considering animal transfer to and from the scanner as well as setup and planning sequences, scanning times in sheep are not recommended to exceed 4 h to ensure maximum image quality and animal welfare. Therefore, tailoring of imaging sequences resulting in shortening of scan time became necessary for in vivo experiments (see Table 8.1), unavoidably resulting in a compromise between image quality and sequence usability.

A two-step approach was chosen to consider this situation. First, the detection limit for VSOP-labeled MSC was assessed in the healthy sheep brain after stereotaxic injection of a defined cell number. In the second step, potential cell homing to the ischemic brain after intravenous MSC injection was investigated.

Frameless stereotaxic transplantations were planned and conducted using the Brainsight™ stereonavigation system as previously described in detail (Frey et al. 2004). The system was slightly modified by the supplier to fit special anatomical characteristics of the ovine skull. Six sheep were used in this experiment. A three-dimensional T1 MR dataset of the brain (minimum resolution $1 \times 1 \times 1$ mm) including fiducial markers and a time-of-flight MR angiography (to detect major vessels) were obtained 1 day before surgery. The fiducial markers were fixed to a maxillary splint which is adaptable to the individual shapes of the maxillary molars and the hard palate, allowing a precise and reproducible positioning for each investigated animal. MR imaging was completed within an hour. Two trajectories for stereotaxic cell administration, one in each hemisphere, were planned avoiding close spatial proximity to blood vessels (exception: animal 1, see below). The white matter of the corona radiata was the preferred target location due to its relatively homogenous MRI signal. Predefined numbers of VSOP-labeled cells were injected according to Table 8.2. Labeling was done using 3.0 mM VSOP, and labeling efficacy was confirmed by relaxometry as described above (Sect. 8.2).

The stereotaxic implementation of the cells was performed without complications in all subjects, and maximum deviation between planned and realized trajectory was 1.0 mm in each dimension. In animal 1, serving as a control subject, 50 μL PBS not containing VSOP-labeled MSC was injected into the left hemisphere, and a hemorrhage was induced contralaterally by targeted damage of a small arterial blood vessel.

Locally transplanted animals were subjected to one MRI investigation using a T2* sequence which had been designed as a compromise of image quality and

Table 8.2 Cell deposits in sheep receiving local stereotaxic cell transplantation

Subject no.	1	2	3	4	5	6
Left hemisphere	PBS	100,000	100,000	10,000	10,000	10,000
Right hemisphere	Hemorrhage induced	1,000	1,000	500	500	500

examination time (for imaging parameters refer to Table 8.1). Imaging series were analyzed by three blinded investigators in accordance to the gel phantom assessment.

Injection of PBS did not result in any detectable signal extinction (Fig. 8.3a), whereas induction of moderate hemorrhage resulted in a clear signal loss (about 54 × 23 mm) (Fig. 8.3b). 100,000 VSOP-labeled MSC appeared as an ellipsoid hypointensity (about 60 × 55 mm in its maximum extension), which could clearly be discriminated from surrounding brain tissue (Fig. 8.3c). Signal loss was visible on 15 slices in T2*-weighted images. Transplantation of 10,000 cells resulted in a circular but smaller area of signal extinction (about 3–4 mm in diameter, Fig. 8.3d), which was visible on 10 slices. A small but reproducible hypointensity with an average diameter of less than 2 mm was visible on 5 consecutive slices after transplantation of 1,000 VSOP-labeled MSC (Fig. 8.3e, white arrow head). However, transplantation of 500 cells did not lead to identical signal changes. A punctual hypointensity was found in the assumed area of the cell deposits in two out of three animals (Fig. 8.3f, white arrow head). In those two animals, subsequent histological analysis revealed microbleedings in the area of the cell deposit, which cannot be excluded as the origin of the signal extinctions. Microbleedings were not detected in the macroscopic tissue inspection (refer to Sect. 8.4 for details). Therefore, the in vivo detection limit of stereotaxically transplanted cells was defined at 1,000 cells at 3.0 T in T2*-weighted sequences in the healthy brain.

In the next step, we tested the hypothesis, whether VSOP-labeled MSC actively migrate toward an ischemic lesion. MSC were reported to mediate a multitude of beneficial effects following stroke and were shown to survive in the recipient brain for up to 1 year after intra-arterial administration (Shen et al. 2007a). Homing of MSC is presumed to be major element of the therapeutic effect by many investigators and was reported to depend on the SDF-1/CXCR4 system (Shichinohe et al. 2007). However, these mechanisms were only observed in rodent species; no evidence exists whether these effects can be reproduced in larger animals or human patients. This may be of critical relevance as, for instance, larger brain volumes and thereby longer traveling distances may hamper MSC homing toward and within the ischemic brain in large animal species and humans.

Transcranial permanent full cortical middle cerebral artery occlusion (MCAO) resulting in a large territorial infarct was induced in three Merino rams as described previously (Boltze et al. 2008). A brief description of the surgery is given in Fig. 8.4. The dose of administered VSOP-labeled cells was set to 1×10^6 per kilogram bodyweight. Cells were administered intravenously 24 ± 1 h after MCAO.

Sheep were subjected to 3.0 T MRI 24 h following MSC injection (48 h after MCAO) as well as 3 and 7 days thereafter. A short T2-weighted turbo spin echo

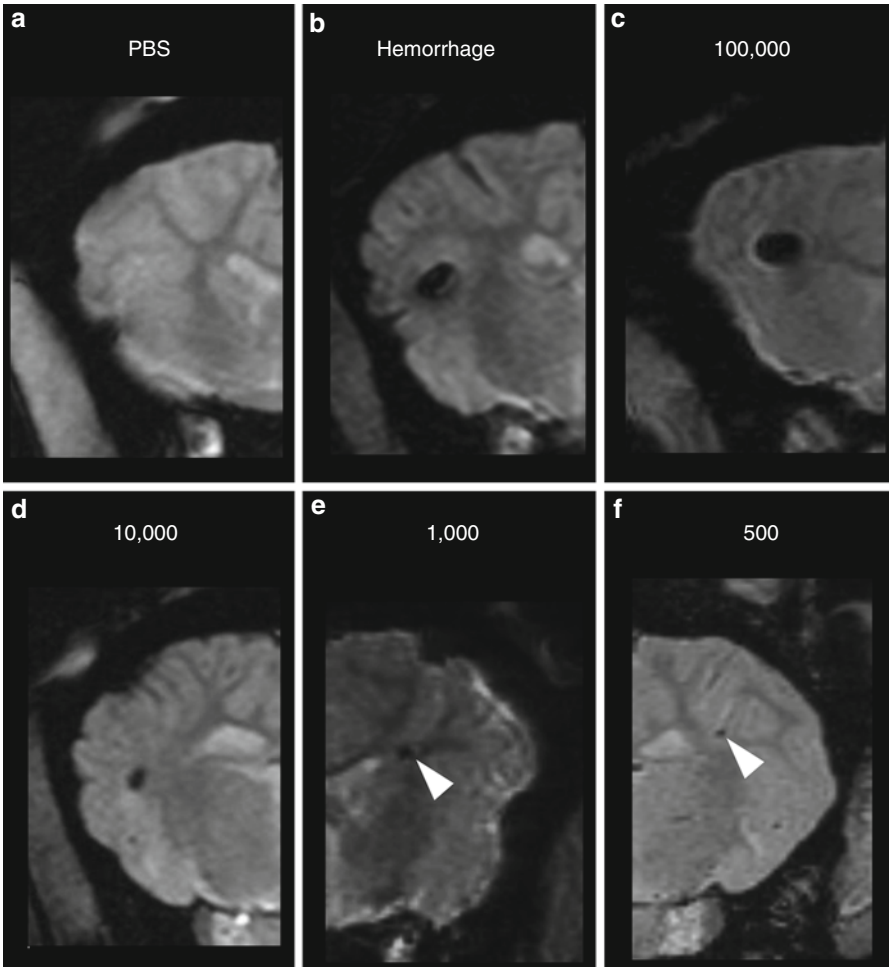

Fig. 8.3 Detection limit of VSOP-labeled autologous ovine MSC after stereotaxic cell administration at 3.0 T. Deposits containing a solution of VSOP-labeled, autologous MSC or PBS were injected stereotactically in the sheep brain (see Table 8.2). Brains were scanned using a T2* sequence (see Table 8.1). Injection of PBS did not result in any signal voids (**a**). A local hemorrhage, induced for control purposes, was visible as an irregular (ellipsoid-shaped) volume of signal loss (**b**), but was well discriminable from a cell deposit by histological investigation (see Sect. 8.2 and Fig. 8.4). 100,000 locally injected cells were clearly detectable at 3.0 T as a spherical signal extinction (**c**). The volume of the signal extinction became smaller after administration of 10,000 (**d**) or 1,000 cells (**e**, *white arrow head*). After injection of 500 cells, very small local signal extinction were seen at 3.0 T in 2 out of 3 subjects (**f**, *white arrow head*)

sequence (TSE, voxel size 0.55×0.55×2.5 mm, scanning time 6 min) was performed to visualize the stroke lesion (Fig. 8.5a), followed by a T2*-weighted sequence (see Table 8.1). A well discriminable and circumscribed hyperintense diffusion disturbance became evident in the supply area of the left middle cerebral

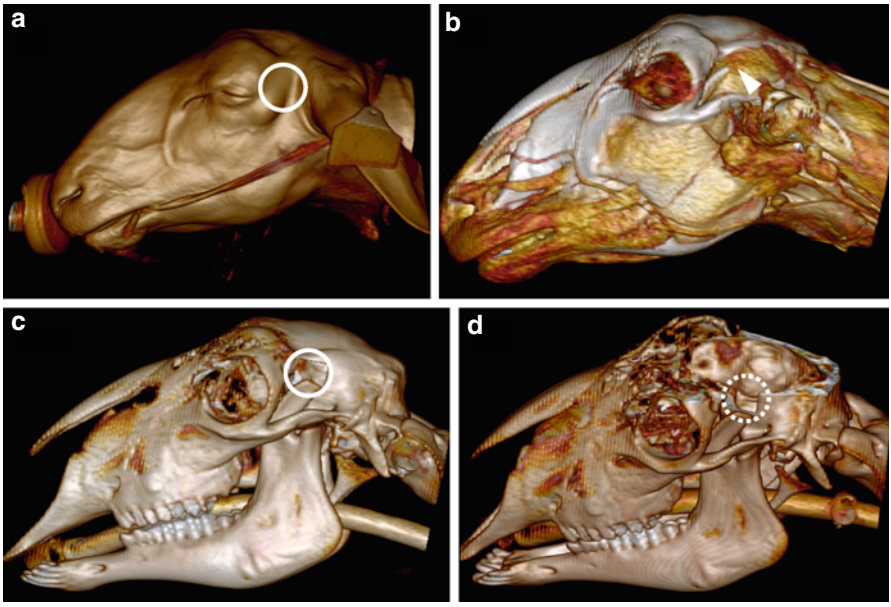

Fig. 8.4 Middle cerebral artery occlusion in sheep. Depicted, three-dimensional images of the sheep head and skull were reconstructed from a previously obtained computer tomography data set. Due to anatomical reason (paunch topography), the field of surgery must be on the left side of the head (**a**, *white circle*). After a skin incision, the temporal muscle (**b**, *white arrow head*) was elevated from the parietal skull bone. Subsequently, a craniotomy (**c**, *white circle*; **d**, *white dotted circle*) was performed using a high speed surgical burr. The middle cerebral artery was occluded by electrocauterization after local incision of the dura

artery in all subjects 2 days after MCAO (Fig. 8.5a). The area of ischemic damage mainly comprised the cortex and the white matter of the temporal lobe but also the claustrum and lateral areas of the external capsule as well as of the putamen. The lesion caused a clearly visible midline shift due to significant cytotoxic edema (Fig. 8.5b). It displaced normal radiological signs of reorganization and receding edema at day 3 (Fig. 8.5c) and day 7 (Fig. 8.5d) following MCAO. No further correlates for pathological events or processes could be observed in the TSE sequences. Furthermore, no hypointensities clearly attributable to VSOP-labeled MSC in the lesion or bordering areas in T2*-weighted imaging could be found (Fig. 8.5e–g) at any time point following MSC injection. Thus, there was no evidence for the homing of VSOP-labeled MSC after intravenous injection from MRI investigations.

8.4 Detection of VSOP-Labeled MSC *Ex Vivo*

Even though no signs for MSC homing toward the ischemic lesion were observed in MRI, minor numbers of migrated MSC below the detection threshold at 3.0 T may have been present in the brain. Moreover, labeled MSC may have migrated

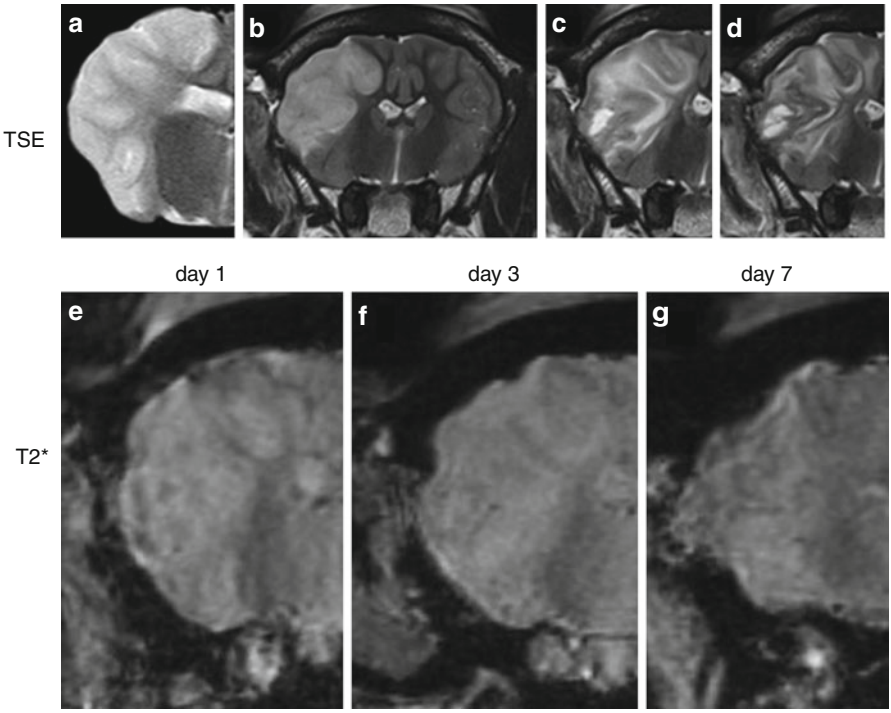

Fig. 8.5 MRI after intravenous injection of VSOP-labeled autologous MSC. A typical ischemic lesion (**a**) causing an obvious brain midline shift (**b**) was clearly detectable 2 days after middle cerebral artery occlusion in all subjects using a turbo spin echo (TSE) sequence. The lesion showed typical signs of reorganization 4 (**c**) and 8 (**d**) days after MCAO. Brain investigation using a T2* sequence according to Table 8.1 did not reveal any signal extinction caused by VSOP-labeled MSC 1 (**e**), 3 (**f**) or 7 (**g**) days after intravenous cell administration.

to other organs which were not investigated by MRI. A pathohistological tissue inspection (macroscopic and microscopic) was performed to reveal such potential events.

The induced hemorrhage induced in animal 1 of the local transplantation group was macroscopically visible (Fig. 8.6a, white dotted circle), whereas the site of PBS injection could not be identified. No signs for tissue damage or other (micro-) bleedings were observed on the macroscopic level after stereotaxic administration of the cells. The injection sites of 100,000 VSOP-labeled MSC could be identified as small brownish areas (Fig. 8.6b, black arrow head), which was not the case for 10,000 or less cells. For microscopic assessments, tissue samples were taken from the areas of stereotaxic injection or along the ischemic lesion, respectively. In MCAO subjects, corresponding tissue samples were also taken from the unaffected contralateral hemisphere and stained with hematoxylin/eosin (HE) or PB. Representative tissue samples were also taken from the sutured MCAO head wound (2× each), lung (4× each), liver, spleen, and kidneys.

All MSC deposits as well as the injection tracks could be identified histologically in serial slices from animals receiving stereotaxic cell transplantation. Locally transplanted, VSOP-labeled MSC could be identified as dense cell agglomerations in the HE stained white matter (Fig. 8.6c). Expectably, number and density of MSC became lower in deposits containing fewer cells. Mononuclear cells, which presumably immigrated to the injection site, were also found in and close to cell deposits. The presence of iron-containing cells was confirmed in PB staining (Fig. 8.6d). In some cases, scattered erythrocytes could be identified among the iron-labeled MSC. Erythrocytes could be clearly discriminated from iron-labeled MSC due to a much smaller size and their typical shape. However, in two out of three deposits containing 500 VSOP-labeled cells, a significant number of erythrocytes were visible next to PB-positive cells (Fig. 8.6e). Therefore, these events were considered microbleedings, which may have partially contributed to dot-like signal extinctions which were observed in MRI in these animals.

Histological investigation of the hemorrhage induced in animal 1 (control) revealed abundant erythrocytes and scattered mononuclear cells. The dimensions of this hemorrhage were comparable between MRI and tissue slices. In contrast, the signal extinction caused by labeled MSC was much larger as compared to the macroscopically visible cell deposit. This phenomenon is known as the blooming effect, being responsible for susceptibility artifacts that exceed the real dimension of an iron-labeled object by a factor of up to 50. The blooming effect, primarily emerging from the continuous biodegradation of hemoglobin into superparamagnetic hemosiderin, is less pronounced in early hemorrhages. Together with time-dependent changes of signal characteristics in hemorrhages (Kidwell and Wintermark 2008), this observation can help to discriminate signal extinctions caused by VSOP-labeled cells from those caused by hemorrhages.

Brain slices from animals that received intravenous administration of VSOP-labeled MSC were carefully examined to identify single PB cells at a 20-fold magnification. Only very few, scattered PB-positive cells (1–6 per slice) could be identified in the leptomeninx and the subarachnoid space (Fig. 8.7a, black arrow heads) of MCAO animals, but not in the brain parenchyma of the lesioned hemisphere (Fig. 8.7b). PB-positive cells could be observed neither in the contralateral leptomeninx (Fig. 8.7c) nor in the brain parenchyma of the contralateral hemisphere. Thus, no relevant homing of VSOP-labeled MSC toward the ischemic brain took place after systemic injection of the cells. PB-positive cells were also absent in the livers (Fig. 8.7d) and kidneys (Fig. 8.7e) of transplanted subjects. PB-positive cells could be indentified in the spleen of all animals (including control spleens from animals which were not subjected to MCAO or administration of VSOP-labeled cells, data not shown), presumably in the red pulp (Fig. 8.7f, white frame and insert). Scattered PB-positive cells were also observed in the white pulp, but only in animals which received MSC. Since the spleen is responsible for filtering and degradation of aged erythrocytes from the circulation, the presence of such cells in the spleen is not surprising. However, a large amount of PB-positive cells was identified in the lungs of all animals which received cell transplantation, but not in control tissue slices. These cells were distributed over the entire organ but mainly appeared in the alveoli

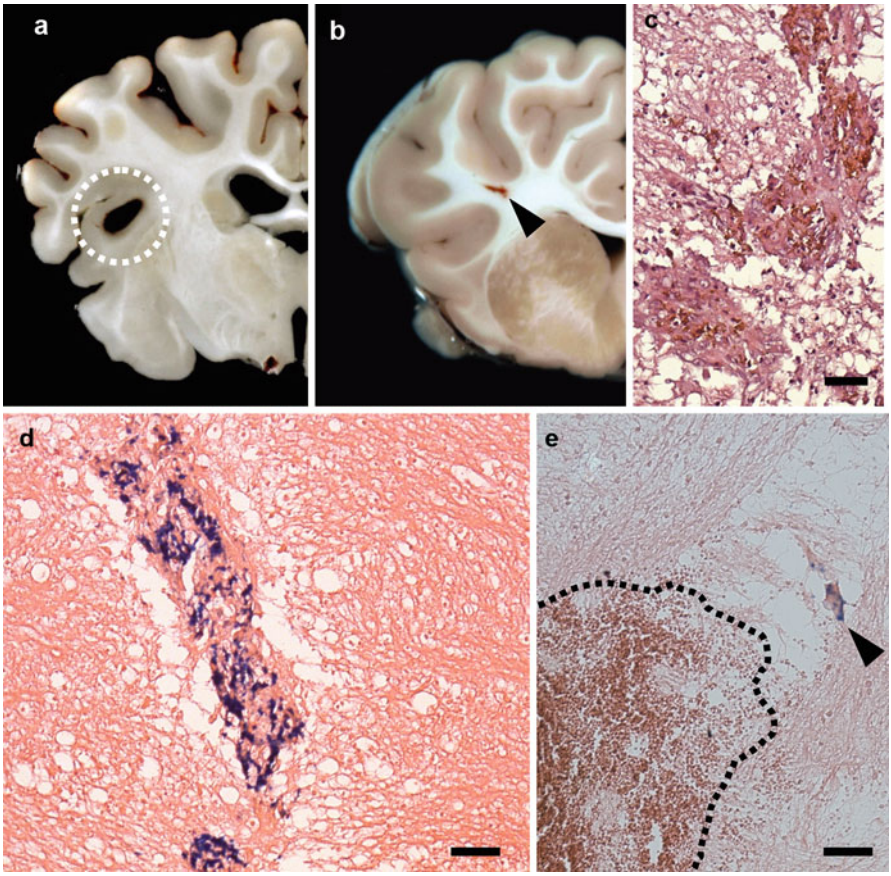

Fig. 8.6 Histological detection of stereotactically administered MSC. The sites of hemorrhage induction (**a**, *white dotted circle*) and the 100,000-cell deposit (**b**, *black arrow head*) were already visible in macroscopic brain slices. Local deposits of MSC were clearly detectable as dense structures in the HE (**c**). Prussian-blue staining (**d**) clearly revealed the presence of iron-containing cells at the site of local MSC deposits. However, in those animals in which signal extinction occurred after local administration of 500 cells, single Prussian-blue-positive cells (**e**, *black arrow head*) were observed in close proximity to small microbleedings in 2 of 3 cases (**e**, main part of microbleeding engulfed by *black dotted line*). Since this may have caused a unspecific signal loss, the in vivo detection limit was defined at 1,000 cells. Scale bars: 50 μm

and in the interstitial connective tissue (Fig. 8.7g), even though the cells could not be discriminated from pulmonary macrophages. This indicates a loss of VSOP-labeled MSC during pulmonary passages, but the amount of cell loss cannot be estimated from the obtained data. Interestingly, PB-positive cells which morphologically appeared similar to myofibroblasts were identified in areas of tissue restoration within the head wound (Fig. 8.7h). Similar cells were neither observed in skin samples from other areas in these animals nor in subjects which were not subjected to administration of VSOP-labeled MSC.

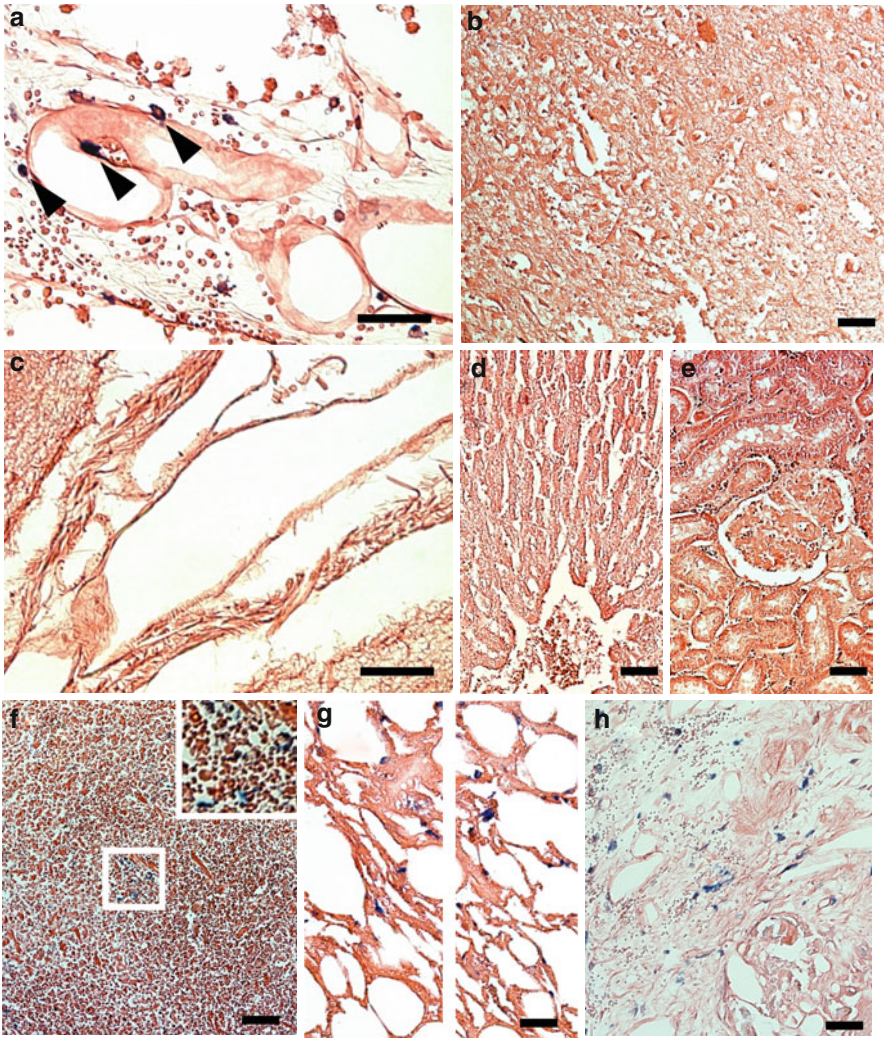

Fig. 8.7 Histological detection of intravenously injected MSC. No iron-containing cells were observed in the ipsilateral (lesioned) or contralateral gray or white matter. Very rarely, scattered Prussian-blue-positive cells were identified in the ipsilateral leptomeninx of MCAO animals (**a**, *black arrow heads*) but not in the ipsilateral brain parenchyma (**b**), the contralateral leptomenix (**c**), or the contralateral brain parenchyma. Further, no cells were observed in the liver (**d**) or kidneys (**e**) of transplanted subjects. Iron-containing, Prussian-blue-positive cells were found in the red pulp of the spleen in all subjects and control tissue samples from animals that did not receive VSOP-labeled MSC (**f**, high-power image of framed area given in *inset*). However, numerous MSC-like, iron containing cells were found in lungs (**g**) and the head wounds (**h**) of cell transplanted subjects. All images depict Prussian-blue stainings. Scale bars: 50 μm

Summarizing, there was no evidence for a target migration of magnetically labeled autologous MSC toward the ischemic lesion after systemic administration. In turn, some evidence was found that a significant proportion of cells got stuck in

the lungs whereas others potentially participated in wound healing processes. A final conclusion about the reasons of lacking cell migration would be preliminary because additional cell labeling techniques (e.g., using fluorescence dyes) or detailed description of the phenotype of PB-positive cells may be required to reveal conclusive information about cell fate.

Conclusions

The beneficial impact of systemically administered bone marrow-derived MSC after focal cerebral ischemia was reported in a number of studies. However, it still remains unclear whether these cells need to be located in areas bordering the ischemic tissue to induce these effects and whether a targeted homing of MSC is possible at all in large animals or humans, taken into account the substantially longer migration distances. To our knowledge, an experimental setup to investigate these questions using large animal models has not been developed up to now. The present study was designed to establish a labeling protocol for ovine MSC and to investigate the feasibility of autologous MSC tracking in sheep using a clinical MRI scanner. The study clearly revealed that ovine MSC can be detected both in gel phantoms and in vivo in the brain using a clinical 3.0 T MRI after VSOP labeling and autologous transplantation. Consequently, identification (and conceivably tracking) of labeled MSC by MRI in the sheep brain can be performed using a 3.0 T clinical scanner, without side effects of labeling procedure. Notably, our study revealed findings not reported in small animal models. The homing of bone marrow-derived MSC to the ischemic brain was repeatedly reported in rodent experiments (Chen et al. 2001; Wu et al. 2008). In contrast, in our experiments a significant migration toward infracted tissue was not observed.

Applying T2*-weighted sequences, the detection limit for MSC was found to be 500 cells in gel phantoms and 1,000 after local stereotaxic cell transplantation. Similar differences were also found in small animal models (Stroh et al. 2005) and are at least in part attributable to the compromise between image quality on the one side and demands of imaging setup in animals (particularly scan time but also available coils as well as scanner hard- and software configuration) on the other. Thus, alternative imaging sequences allowing for faster scan performance at same or even higher sensitivity for susceptibility differences or changes as compared to T2*-weighted sequences may be demanded. Moreover, sequences particularly prone to field inhomogeneities such as T2*-weighted sequences may require custom-built coils and elaborated nonlinear shimming, still limiting cell detection in common T2* sequences (Pillai et al. 2011; Denic et al. 2011).

Susceptibility-weighted imaging (SWI) is characterized by a very high sensitivity for magnetic field distortions caused by paramagnetic and ferromagnetic substances, leading to negative contrast signal voids. The sensitivity of SWI sequences is discussed to be significantly higher compared to T2*-based imaging (Sehgal et al. 2005; Dai et al. 2011), but discrimination between hemorrhages and VSOP-induced signal changes remains difficult with SWI sequences (Haacke et al. 2009). Nevertheless, an assessment of the applicability of SWI for MSC tracking in the large animal brain including a direct comparison to a T2*-weighted sequence should be performed in future experiments, particularly for

the detection of comparatively small numbers of labeled cells that migrated to the lesion site and may only be detectable with an optimized imaging setup. However, MSC could be identified using T2*-weighted MRI after local injection also in relatively small numbers of 1,000 MSC in a deposit. Moreover, no indications for the presence of iron-loaded cells in brain parenchyma were observed after systemic administration. Insufficient field strength and/or sequence parameters being unable to detect minor cell numbers are therefore rendered unlikely to be the sole reason for lacking cell detection by MRI. More likely, there was only a very limited or even no migration of autologous MSC to the ovine brain.

The number of cells injected in this study was 1×10^6 per kg bodyweight. Usually, between 1×10^6 (e.g., Chen et al. 2003) and 3×10^6 (e.g., Okazaki et al. 2008) are reported to be administered in a rat, with 3×10^6 cells being reported effective (Chen et al. 2001). The average weight of adult laboratory is strain-dependent but can be found in the range of 300–450 g. Thus, the number of cells injected into sheep is relatively small, but a histologically detectable number of MSC should have reached the brain parenchyma also after administration of a "subtherapeutic" MSC dose. Moreover, iron-containing cells were detected in other tissues including the leptomeninx so an insufficient cell number for administration is unlikely the cause for unsuccessful detection of cell homing to the ischemic lesion (Sect. 3). The SDF-1/CXCR4 system, which is reported to mediate MSC migration to an ischemic lesion (Shichinohe et al. 2007), is also relevant for attracting immune cells during the inflammatory as well as the proliferative phase of wound healing processes in numerous tissues including the skin (Shichinohe et al. 2007; Ding et al. 2011; Hannoush et al. 2011; Fujio et al. 2011). In fact, iron-containing cells were identified in the skin wounds of animals of MSC-treated subjects. These cells were observed in skin wound samples from animals which did not receive VSOP-labeled cells. This indicates that a targeted homing of MSC to a side of injury is in principle possible. It may also be hypothesized that the migratory stimulus emerging from the head wound may competitively interact with the one resulting from the ischemic brain lesion.

Numerous iron-loaded cells have been found in the ovine lungs after intravenous cell administration. The identity of these cells is hard to unambiguously assess in an autologous setup, but similar cells were not found in untreated subjects. It may be assumed that these cells are in fact injected MSC since immunologic rejection or destruction of autologous MSC, even after *ex vivo* processing, has not been reported so far. Moreover, a "filter effect" of the lungs for MSC, but not for mononuclear cells, was reported in a rodent study (Fischer et al. 2009). This is reasonable considering the fact that MSC (1,000–7,000 μm^3) are significantly larger than MNC (100–350 μm^3). Further assuming MSC being spheroid entities when traveling in the blood stream, the cells would have a diameter between 12.4 and 23.7 μm. This is by far larger than the average diameter of pulmonary capillaries (5–10 μm), which may contribute to the filter effect even though MSC cannot be considered "rigid" cells.

Given the reported benefit of MSC administration after stroke, additional strategies will most likely be required to enhance the number of MSC homing to the cerebral lesion in order to improve the therapeutic impact. The impact of this

alternative administration rout needs to be investigated in this particular model. For example, intra-arterial cell delivery may help to reduce the amount of MSC filtered from the lungs, at least during the first passage after cell injection. Additionally, the influence of strategies aiming to enhancing migration capabilities of MSC to lesioned areas by cell surface engineering (Sarkar et al. 2011a, b) will have to be assessed in further studies.

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Neural Stem Cell Mapping with High-Resolution Rapid-Scanning X-Ray Fluorescence Imaging

9

Angela M. Auriat, Helen Nichol, Michael Kelly, and Raphael Guzman

9.1 Introduction

In pre-clinical studies, cell-based therapies improve functional outcome in a variety of diseases of the central nervous system (Chopp et al. 2008). Many different types of stem cells, including bone marrow stromal (BMS) cells, embryonic stem (ES) cells, fetal neural stem (FNS) cells, and umbilical cord blood cells, have been tested (Bliss et al. 2010). Human trials indicate that stem cell treatments are safe and well tolerated (Nelson et al. 2002; Savitz et al. 2005; Kondziolka et al. 2000, 2004, 2005). However, before clinical cell transplantation becomes mainstream, the ideal route and time of delivery, as well as the mechanisms of action need to be identified. Such studies would benefit from serial long-term imaging of transplanted cells with high spatial resolution, sensitivity and functional information. The technique should not impact the therapy, having no influence on cell differentiation, survival, physiology, migration, or mechanisms of action. Additionally, the ideal imaging tool would be able to differentiate viable from dead cells. Several technologies have been used for in vivo neural stem cell imaging, including MRI, positron emission tomography (PET), optical imaging, and single-photon emission computed tomography (SPECT). The relatively high spatial resolution, sensitivity, availability, and lack of

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ionizing radiation has made MRI one of the most frequently used imaging modalities for in vivo stem cell tracking.

For MR imaging, cells must be preloaded with a contrast agent, such as gadolinium-rhodamine dextran (Modo et al. 2004), superparamagnetic iron oxide (SPIO) (Arbab et al. 2004; Guzman et al. 2007), or ultrasmall superparamagnetic iron oxide (USPIO) particles (Guzman et al. 2008; Bulte et al. 2002). The most common technique for labeling cells is using SPIO in combination with a transfection agent such as Lipofectamine or protamine sulfate. The SPIO strongly affects the T_2 relaxation time, resulting in hypointensities on the MR images. Several studies have used MRI to longitudinally track transplanted iron-labeled cells in different animal models including stroke (Modo et al. 2004; Guzman et al. 2007, 2008; Bulte et al. 2002; Hoehn et al. 2002; Zhang et al. 2003; Franklin et al. 1999). However, if transplanted cells divide or migrate away from each other, the SPIO signal becomes diluted (Berman et al. 2011). In addition, since SPIO is inert it is not destroyed when stem cells die or are phagocytosed by macrophages after transplantation (Bliss et al. 2007). Thus SPIO can be detected with MRI long after all transplanted cells have died (Berman et al. 2011). Moreover, many parts of the brain are naturally rich in paramagnetic iron (ferritin and hemosiderin) which cannot be distinguished from SPIO with MRI. These issues raise concern over the usefulness of long-term MRI tracking of SPIO-labeled cells following transplantation.

Synchrotron rapid-scanning X-ray fluorescence mapping (RS-XRF) can both map and quantify total iron in tissues, but it must be used with discretion. Quantification of iron in individual histological sections will allow for the study of changes in SPIO concentration. The following review will identify the key strengths and weaknesses of using RS-XRF to identify SPIO-labeled stem cells, will outline the current findings, and lastly indicate the future potential for this technology.

9.2 Cell Tracking with SPIO

9.2.1 Issues with Tracking SPIO-Labeled Cells

Cell death and division are two major issues when imaging SPIO-labeled stem cells. In a recent study by the Walczak group, SPIO-labeled stem cells were injected into the brains of immunocompetent and immunodeficient mice (Berman et al. 2011). Serial MR imaging of the transplanted cells showed a more intense and persistent signal detection in the immunocompetent mice, in which no surviving transplanted cells could be identified. Whereas, the immunodeficient mice had a more rapid reduction in MR T2 signal detection over time, corresponding to a rapid proliferation and migration of transplanted cells, which was confirmed with bioluminescence imaging and immunohistochemistry.

There is conflicting evidence regarding the degree of clearance/persistence of SPIO signal following the death of labeled cells. Guzman et al. (2007) showed

that SPIO-labeled NSCs killed with repeated freeze-thaw cycles prior to injection were nearly completely cleared, whereas the living cells injected into the contralateral hemisphere remained detectable. Similar findings were also described when using rodent neural stem cells (Zhang et al. 2003). When SPIO-labeled cells are injected directly into the cisterna magna, no iron-positive cells were detected with Prussian blue staining, suggesting that all iron from the labeled cells is cleared (Zhang et al. 2004). However, other studies have found a persistent MRI signal after the death of transplanted SPIO-labeled cells (Winter et al. 2010; Gonzalez-Lara et al. 2011).

Previous research has shown that some cell lines undergo asymmetric cell division, resulting in a sharp drop-off in SPIO label in one population of cells and not in another (Walczak et al. 2007). In this study, SPIO-labeled C17.2 cells were injected into the lateral ventricle of neonatal shiverer mice; the cells retaining the most SPIO remained at the lateral ventricles, whereas the cells with less SPIO migrated away from the ventricles and rapidly had undetectable levels of iron labeling. Quantification of iron in individual cells with XRF would allow for precise measurements of the loss of iron in migrating cell populations.

MR imaging of SPIO-labeled cells is impaired in injury models with a strong inflammatory response (Vandeputte et al. 2011). In the photothrombotic model of stroke, T2*-weighted images have hypointensities resulting from the accumulation of endogenous iron containing inflammatory cells and glial scar formation at the border of the injury. The images from the non-cell-treated animals were indistinguishable from those treated with SPIO-labeled stem cells (Vandeputte et al. 2011). The combination of RS-XRF and immunohistochemistry could allow for the differential quantification of iron in inflammatory and SPIO-labeled transplanted stem cells.

9.2.2 Tracking Cells After Different Methods of Transplantation

Intravascular and stereotactic cell transplantation are two major methods of stem cell transplantation (Pendharkar et al. 2010). Stereotactic transplantations inject the cells directly into the brain, either parenchyma or intracisternally. Intravascular transplantations inject cells into the periphery either intra-arterially, typically into the blood vesicles supplying the brain, or intravenously. Each transplantation method has a unique pattern of cell distribution and engraftment (Pendharkar et al. 2010). Several studies have compared the different transplantation methods, although generalizations across these studies are difficult as there are differences in injury models, cell type, treatment delay, and other factors that can alter distribution and engraftment results (Auriat et al. 2011). Comparison of intraventricular, intraparenchymal, and intravenous transplantations indicated that the greatest number of cells engrafted in the brain is found following the intraparenchymal injection (Jin et al. 2005). If we compare just the vascular delivery methods, intra-arterial injections have a far greater engraftment of NPCs in the ischemic brain than intravenous

injection (Pendharkar et al. 2010; Li et al. 2010). Similar results have been found after intra-arterial compared to intravenous transplantation of mesenchymal stem cells (Walczak et al. 2008). The differences in engraftment and distribution with different transplantation methods have implications for the ease with which cells can be detected with MRI. SPIO-labeled stem cells, transplanted in a variety of ways, have been identified in the ischemic rat brain with MRI (Guzman et al. 2007; Li et al. 2010; Walczak et al. 2008). However, with diffuse distribution after intravascular delivery, more sensitive monitoring is required. The cellular resolution and high sensitivity of RS-XRF make it ideal for identifying small quantities of iron in SPIO-labeled stem cells.

9.3 Rapid-Scanning X-Ray Fluorescence Mapping

X-ray fluorescence is a quantitative technique for mapping element distribution. Other established techniques can quantify element concentrations at low levels with high accuracy such as inductively coupled plasma mass spectrometry (ICP-MS) and atomic emission spectroscopy (AE). However, these technologies require the isolation and purification of the target structure; this can be a difficult task and may result in contamination artifacts. Recent developments with third-generation synchrotrons, which can generate spatially coherent high-brilliance X-rays, have resulted in the ability to quantify elements nondestructively, allowing for the mapping of elements in hydrated tissue sections and whole cells with high sensitivity and micron resolution (Paunesku et al. 2006). The high sensitivity of this technology, both in terms of localization and quantification, make it ideal for studying iron in transplanted stem cells.

9.3.1 Basic Principles of X-Ray Fluorescence

A tissue slice or whole mount is raster scanned in a collimated beam of hard X-rays having an energy above that needed to eject core-shell electrons from all elements of interest (Fig. 9.1). A higher shell electron fills the electron hole resulting in the emission of a photon equal to the difference in binding energies of the two shells involved. Each binding energy is proportional to the squared nuclear charge, meaning that the emitted photon energy is unique for each element. The emitted photons are detected and used to identify and quantify the elements present in the sample. The X-ray beam can be focused allowing for high-resolution images (Fahrni 2007).

Rapid-scanning X-ray fluorescence mapping (RS-XRF) is a new imaging technique developed at the Stanford Synchrotron Radiation Lightsourse (Popescu et al. 2011). The primary advantage of rapid scanning is that large samples can be mapped in a reasonable time. Emission spectra are collected at each point and by selecting the appropriate energy ranges, multiple elements can be mapped simultaneously. The X-ray fluorescence counts can be further quantified by comparison with XRF standards.

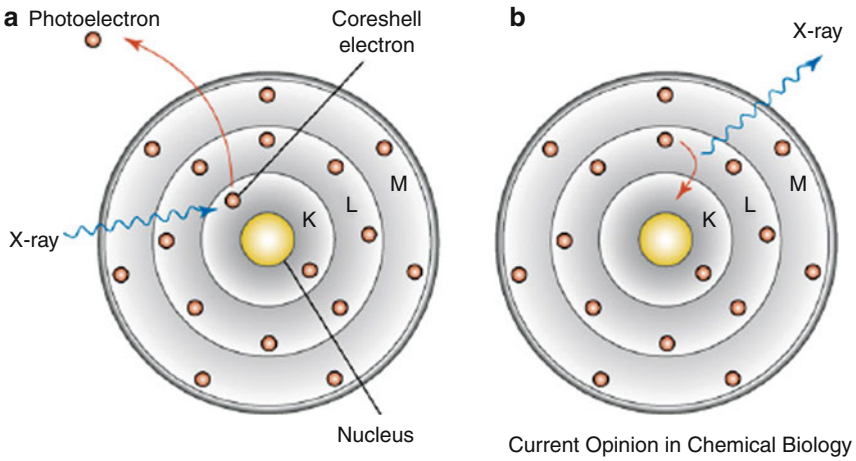

Fig. 9.1 Bohr atom model. X-ray fluorescence imaging involves the excitation of the sample with an X-ray, leading to the ejection of a core-shell electron from the atom (**a**). An electron from a higher shell falls down to fill the electron hole, resulting in the emission of a photon of an energy equal to the difference in binding energies of the two shells involved in the transition (**b**) (Reprinted from Fahrni (2007) with permission from *Current Opinion in Chemical Biology*)

9.3.2 Biological Applications of XRF

Previously, XRF has been used in the mapping of clinical postmortem tissue in various patient populations (Szczerbowska-Boruchowska et al. 2011, 2012) and in animal models (Chwiej et al. 2011). Of particular interest are neurodegenerative diseases, in which irregular distributions of various elements are thought to be important. For instance, abnormal metal distribution of elements such as Cu, Fe, and Zn have been suggested to play a role in neurodegenerative disorders including Alzheimer's disease, Parkinson's disease,and amyotrophic lateral sclerosis. Metal deposits can be nondestructively mapped in brain tissue and individual cells (Tomik et al. 2006; Chwiej et al. 2005; Szczerbowska-Boruchowska et al. 2012), and relationships between metals can be easily identified with colocalization and quantification. Animal models can also be assessed with RS-XRF to identify element distribution following brain injury (Silasi et al. 2012; Auriat et al. 2012). Sensitive mapping and quantification of metals can be particularly useful in the assessment of treatments, such as therapeutic chelators, which alter metal levels in the brain (Auriat et al. 2012; Popescu and Nichol 2011). Quantitative and topographic mapping of element distributions with RS-XRF would be particularly useful for identifying how specific the chelators are as well as how chelation of one metal may alter the distribution of other metals. At the cellular level, XRF has been used to examine the mechanisms of pluripotency and differentiation in embryonic and induced pluripotent stem cells (Cardoso et al. 2011). Elemental maps at the atomic level indicated that phosphorus and sulfur levels rise and consistent patterns of element polarization within the cells are observed during neural differentiation.

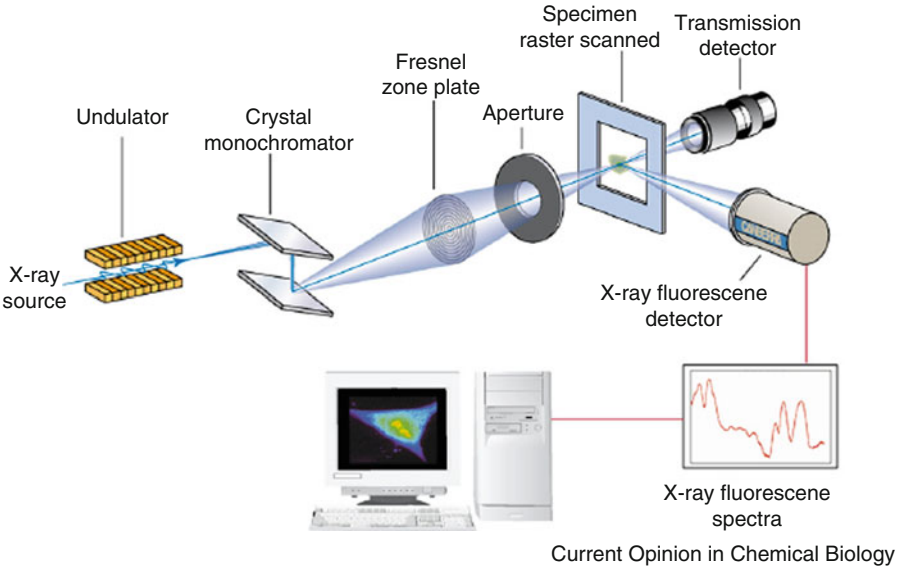

Fig. 9.2 Illustration of one of the typical sample setups used in RS-XRF. The crystal monochromator is used to select the desired X-ray energy, which is then focused with a Fresnal zone plate. Apertures of varying sizes are used to control the spot size of the beam. The sample is moved through the beam; this raster scanning across the area of interest results in a quantitative element map (Reprinted from Fahrni (2007) with permission from *Current Opinion in Chemical Biology*)

9.4 Sample Preparation

The high sensitivity of XRF makes samples highly sensitive to contamination. Throughout the processing of tissue samples, care must be taken to avoid introducing any foreign elements. Solutions used to process samples should be prepared with ultrapure water (Auriat et al. 2012; Hackett MJ et al. 2012). For the study of SPIO-labeled stem cells in tissue, brains can be fixed and cryostat-sectioned using a Teflon-coated blade. Sections should be placed on metal-free plastic coverslips such as Thermanox. Our group has previously imaged blank Thermanox coverslips and found them to be low in all elements of interest for our samples. It is also critical to keep sectioned tissue in an atmosphere free of dust and other contaminants, because any partials on the samples will be observed in the resulting image (Fig. 9.2).

9.5 Cellular Iron Quantification in SPIO-Labeled Stem Cells

Our recent findings indicate that it is possible to use RS-XRF to characterize the migration of SPIO-labeled neural stem cells and to correlate these findings with MRI.

The scanning was completed at the Stanford Synchrotron Radiation Lightsource (SSRL) on beamline 2–3. The 13-keV beam was oriented at 45° to the vertically mounted samples and 90° to the detector. Sections from the injured hemisphere were imaged at up to a 3-μm resolution with a 200-ms dwell time, allowing for the identification of individual cells. Quantification was completed by comparing the signal intensity of the samples to the signal from standards of known concentrations (Micromatters Inc., Sault Ste. Marie, ON, CAN). Analysis of the SPIO-labeled hNPCs at high resolution showed that on average stem cells contained about 7 pg of iron (Fig. 9.3).

The nondestructive nature of RS-XRF imaging means that after imaging, the same sections can be labeled with immunohistochemical markers. Recent advances at SSRL now allow for simultaneous mapping of fluorescent immunohistochemical markers and RS-XRF imaging, facilitating the identification of specific cell types.

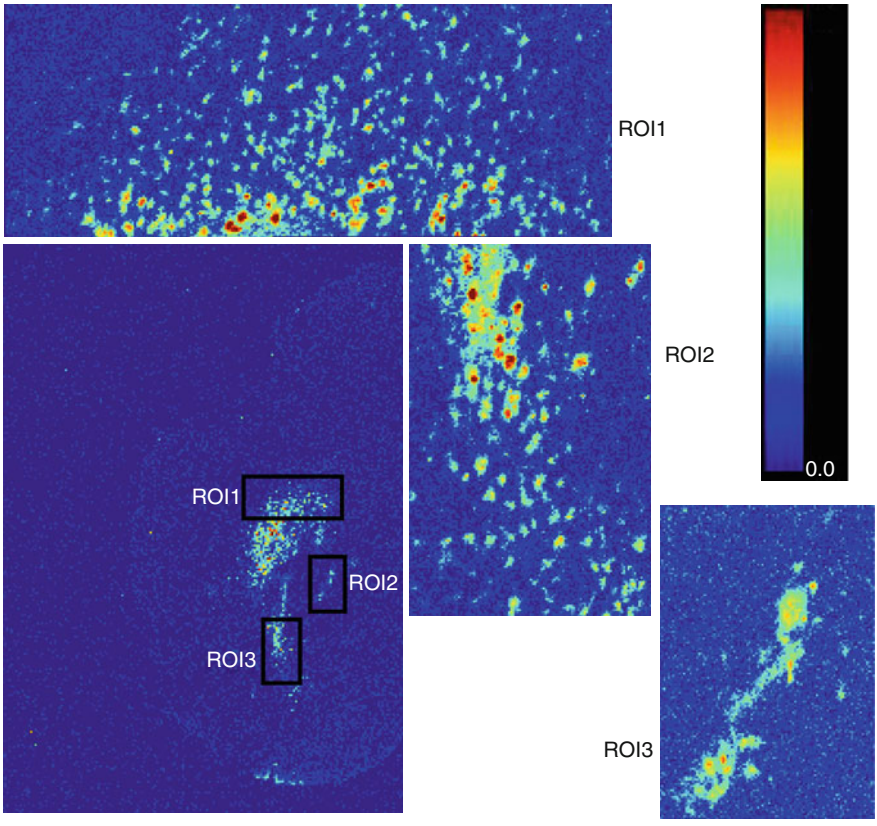

Fig. 9.3 RS-XRF image of SPIO-labeled stem cells injected stereotactically into an ischemic mouse brain. The high-resolution ROI correspond to the areas identified in the ischemic hemisphere. Concentrations of iron are color coded, with red being the highest level and blue being the lowest. The color legend for XRF images represents pg/cm^2 iron

9.6 Future Directions for RS-XRF

The synchrotron rapid-scanning XRF imaged cells at high resolution and allowed for iron quantification in individual cells. These are highly promising results, indicating that XRF will be useful for future studies identifying parameters important to monitoring SPIO-labeled stem cells in vivo. With the ability to accurately map and quantify iron levels in individual cells, it will be possible to determine the concentration of iron in different populations of transplanted stem cells. The development of genetically encoded reporters, whereby specific protein expression causes the formation of suitable contrast agents, will likely be important in future research. Endogenous and persistent generation of cellular contrast would be highly beneficial for studies of stem cell transplantation, ensuring the specific and persistent imaging of surviving cells. Several MRI reporter genes are being developed, including those expressing iron homeostasis proteins such as transferrin receptor (Weissleder et al. 2000) and ferritin (Genove et al. 2005; Cohen et al. 2005), as well as the use of the genes present in magnetotactic bacteria (magA) (Zurkiya et al. 2008; Goldhawk et al. 2009). RS-XRF could play an important role in the development of new reporter genes, accurately quantifying the iron signal in individual cells, helping to ensure that the threshold for MRI detection is met.

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In Vivo Biodistribution Studies and Cell Tracking in Stroke Using SPECT Imaging

10

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10.1 Introduction

Molecular imaging provides a powerful tool for the characterization and measurement of biological processes at a molecular level in living subjects (Weissleder and Mahmood 2001). Noninvasive imaging methods are based on the detection of signals that originate from labeled molecules in order to observe their interactions with specific cellular targets or molecular pathways over time *in vivo*.

Most imaging techniques were originally developed for human use and are now routinely used clinically, both as diagnostic tools and for the evaluation of therapeutic efficacy. However, recent technological advances, especially in terms of spatial resolution, have led to the development of dedicated small animal devices that are able to image the biodistribution of molecular probes with high sensitivity and resolution (Koba et al. 2011). Sophisticated small animal imaging technologies can greatly extend preclinical research beyond conventional *ex vivo* animal studies and allow longitudinal whole body measurements without the need to sacrifice a large number of experimental animals. Follow-up of various biological processes, such as cell trafficking after transplantation, would be difficult without the help of modern noninvasive imaging techniques (Gera et al. 2010).

Single-photon emission computed tomography (SPECT) is based on external detection of high-energy γ-photons emitted by radiolabeled molecules administered to the target. The system typically consists of a gamma camera featuring one or several scintillation detectors coupled to an array of photomultiplier tubes (PMT)

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(Anger 1958; Khalil et al. 2011). During scanning, detector elements rotate around the subject to collect projection images at different angles. The scintillation crystal material is most often thallium-activated sodium iodide (NaI(Tl)), which has the ideal properties for the energy discrimination of γ-photons from technetium-99m (^{99m}Tc), indium-111 (^{111}In), and iodine-123 (^{123}I), the most common radionuclides used for SPECT. The detector converts γ-rays emitted from the subject into visible light, and PMTs further convert light pulses into electrical signals. Finally, the projection data is digitized and reconstructed into tomographic three-dimensional images by using mathematical algorithms based on either filtered backprojection or iterative methods.

Due to the isotropic nature of γ-emission, lead plates or apertures with holes, a.k.a. collimators, are placed between the subject and detectors. The collimators control the paths of photons by allowing only γ-rays coming from a specific direction to enter and interact with the detector crystal. The walls around the collimator holes absorb photons that are not traveling at an angle perpendicular to the collimator. In clinical settings, the most common form of such a system is a parallel-hole collimator. In small animal SPECT with mice and rats, however, pinhole collimation is frequently used (Franc et al. 2008). This can be achieved by either incorporating novel collimator systems into clinical cameras (Beekman et al. 2005; Schramm et al. 2003) or by designing dedicated small animal scanners with compact high-resolution detectors (Weisenberger et al. 2003; Kastis et al. 2004; Furenlid et al. 2004). The use of pinhole collimation improves spatial resolution by producing a magnified projection of the subject onto the detector. However, since the hole in such apertures is rather small, the radiation flux that can reach the detector is restricted, resulting in decreased detection efficiency. Therefore, high-sensitivity SPECT devices have been developed. Instead of using just a single pinhole, these systems are based on, for example, a coded-aperture collimation in which multiple pinholes create overlapping images on the detectors (Meikle et al. 2002; Smith et al. 2003). By using many small holes, a trade-off between resolution and sensitivity can be eliminated.

10.2 Different Imaging Modalities

There have been significant improvements in the designs of small animal SPECT systems over recent years, and several commercial systems are currently available with spatial resolutions of around 0.5 mm (Rowland and Cherry 2008). As a result, SPECT can compete with preclinical positron emission tomography (PET). In addition to improved resolution, another advantage of SPECT over PET is the possibility to perform dual imaging, that is, the measurement of two different radionuclides simultaneously. In terms of cellular therapies, dual imaging may enable simultaneous monitoring of biodistribution and functional properties of administered cells. Unfortunately, SPECT suffers from somewhat reduced sensitivity compared to PET, mostly due to the fact that absorptive collimation must be

Table 10.1 Properties of small animal molecular imaging methods

Modality	Detection	Spatial resolution	Advantages	Disadvantages
CT	X-rays	50–100 μm	High resolution	Poor soft tissue contrast
			Short acquisition time	Radiation exposure
			Clinical translation	
MRI	RF waves	25–100 μm	Excellent resolution	Long acquisition time
			Good soft tissue contrast	Low sensitivity
			Clinical translation	Expensive
SPECT	Gamma emission	0.5–2 mm	High resolution	Low sensitivity
			Dual imaging	Radiation exposure
			Good tissue penetration	
			Clinical translation	
PET	Positron emission	1–2 mm	High sensitivity	Short-lived isotopes
			Good tissue penetration	Requires cyclotron
			Clinical translation	Radiation exposure
				Expensive
Fluorescence	Visible and NIR light	1–10 mm	High sensitivity	Poor tissue penetration
			Short acquisition time	Autofluorescence
			HTS	Limited clinical application
			Inexpensive	
Bioluminescence	Visible light	1–10 mm	No background	Poor tissue penetration
			High sensitivity	Requires transfection of cells
			Short acquisition time	Limited clinical application
			HTS	
			Inexpensive	

CT computed tomography, *MRI* magnetic resonance imaging, *SPECT* single photon emission computed tomography, *PET* positron emission tomography, *RF* radio frequency, *NIR* near-infrared, *HTS* high throughput screening

used to define the direction of radiation. In addition, there is a variety of other imaging modalities, each characterized by their strengths and weaknesses in terms of resolution, sensitivity, and measurement properties (Table 10.1). SPECT gives information on metabolic changes, whereas magnetic resonance imaging (MRI) serves to identify morphological changes in small structures to provide soft tissue contrast. MR measurements can provide additional functional information, such as dynamic contrast-enhanced MRI, diffusion-weighted MRI, functional MRI, pharmacological MRI, and MR spectroscopy, without using ionizing

radiation (Cherry et al. 2008). The main advantage of SPECT over MRI is that it can detect tracer concentrations in the picomolar or nanomolar range. In recent years, attention has focused on creating multimodality systems in which two or more different modalities are integrated into the same device (Cherry 2006). The ability to localize regions of radioactive uptake is greatly facilitated by combining SPECT data with structural information obtained with X-ray computed tomography (CT) or MRI.

10.3 Direct and Indirect Labeling Methods

10.3.1 ^{111}In-oxine

The use of ^{111}In-oxine was originally described for the labeling of white blood cells to image inflammatory processes. For experimental studies, its use has primarily been directed toward cell tracking after myocardial infarction (Kraitchman et al. 2005; Caveliers et al. 2007) and to a lesser extent toward monitoring the migratory behavior of cells after cerebral ischemia (Mäkinen et al. 2006; Correa et al. 2007; Lappalainen et al. 2008; Barbosa da Fonseca et al. 2010). The ^{111}In-oxine complex is lipid soluble and thus penetrates cell membranes. When internalized, indium firmly binds to cytoplasmic components. Free ^{111}In or the ^{111}In-oxine protein complex leaks only from dead cells and is then excreted via the kidney or metabolized by the liver and spleen. As with the other imaging modalities, SPECT is unable to distinguish whether a positive signal is coming from surviving cells or cell debris, which has to be confirmed by histology. The cell labeling with ^{111}In-oxine is straightforward and relatively simple. For labeling, cells are suspended in labeling buffer, and ^{111}In-oxine is added. After incubation, the free tracer is removed by centrifugation, and cells are ready for immediate infusion to animals. A high labeling efficiency (25–90 %) seems to partly depend on the ratio between cells and ^{111}In-oxine (Gholamrezanezhad et al. 2009). ^{111}In-tropolone is also used in cell labeling and tracking studies (Bindslev et al. 2006; Yoon et al. 2010). The advantages compared to ^{111}In-oxine have been discussed in detail previously (Dewanjee et al. 1981).

10.3.2 ^{99m}Tc-Hexamethylpropyleneamine Oxime

^{99m}Tc is another commonly used radionuclide used for labeling leukocytes in order to detect sites of inflammation and infection (McAfee et al. 1984). It has a relatively short half-life of 6 h and can therefore be given at higher doses than ^{111}In, resulting in improved image quality. However, the time window for imaging is restricted to approximately 1 day after administration. The most common label for cell studies is ^{99m}Tc-hexamethylpropylene amine oxime (^{99m}Tc-HMPAO), which is primarily used in brain perfusion imaging. It is a lipophilic complex that is reduced to a hydrophilic

complex after becoming trapped within cells. It provides a simple, rapid, and reproducible way to label cells without significant loss of cell viability or biological functions. As a downside, the stability of the ^{99m}Tc-labeled complex is lower compared to ^{111}In-labeled compounds, and therefore leakage of the radiolabel from the cells may occur over time. The trapped form of ^{99m}Tc-HMPAO is soluble, which causes release of ^{99m}Tc to the kidneys and intestine, and this restricts imaging of the abdominal cavity at early time points. Furthermore, excretion of secondary hydrophilic complexes of ^{99m}Tc-HMPAO shows nonspecific bowel, urinary- and gallbladder activity that hinders imaging of gastrointestinal diseases.

10.3.3 Indirect Labeling

The half-lives of most radionuclides allow monitoring of cell fate for only a limited period of time. Labels attached to parental cells do not readily transfer to daughter cells during cell division, resulting in the dilution of signal over time. Further, radioactive labels may alter gene expression patterns, cause DNA damage, and induce apoptotic pathways. Labels can also detach from cells, for example, during phagocytosis, making the interpretation of results difficult. Short imaging time frames and adverse effects can be partly circumvented by using cells transfected with reporter genes. Imaging of transgene expression is usually carried out with optical techniques, but it is also possible with SPECT imaging (Acton and Zhou 2005). The most common reporter gene in this context is herpex simplex virus type 1 thymidine kinase (HSV-TK) as its expression can be carefully followed with SPECT by using an HSV-TK substrate called $^{123/125/131}$I-FIAU, which is a uracil-based nucleoside analogue (Tjuvajev et al. 1996; Dempsey et al. 2006). This application is usually restricted to imaging peripheral organs since probes for HSV-TK do not cross the intact blood–brain barrier (BBB). However, the BBB is often disturbed in several pathological conditions including stroke, making HSV-TK a feasible system for the imaging of some neurological disorders. Another straightforward strategy for reporter gene imaging is human sodium iodide symporter (hNIS), which can be tracked simply with ^{123}I or ^{99m}Tc-pertechnetate ($^{99m}TcO_4^-$). The approach has been successfully used for the imaging of various cell types (Cho et al. 2002; Dwyer et al. 2011; Sharif-Paghaleh et al. 2011). Despite promising results, further knowledge should still be gathered on how gene transfer, transgene expression, or interactions with the reporter probes affect cell viability, proliferation, and differentiation.

10.4 Cell Viability

The main concern when radioactive tracers are used for cell labeling is their ionizing radiation and their potential cytotoxic effects. This holds true particularly with ^{111}In. One should note, however, that nonradioactive indium is also cytotoxic

Table 10.2 Viability of ^{111}In-labeled cells in vitro

Cell type	Tracer	Labeling dose (Bq/cell)	Incubation time (min)	Follow-up time	Comments	Reference
Canine bone marrow mesenchymal cells	^{111}In-tropolone	0.02–3.6	30	Up to 14 days	100 % viability when dose <0.18 Bq/cell	Jin et al. (2005)
Human bone marrow mesenchymal cells	^{111}In-tropolone	0–260	10	1–7 days	No effect on proliferation	Bindslev et al. (2006)
Human ES-derived neural progenitors	^{111}In-oxine	2.5–7.5	20	24 h	No effect on cell viability	Lappalainen et al. (2008)
Human bone marrow mesenchymal cells	^{111}In-oxine	0.76–7.16	20	2, 24, and 48 h; 5, 7, and 14 days	Time- and dose-dependent toxicity	Gholamrezanezhad et al. (2009)
Rat bone marrow mesenchymal cells	^{111}In-tropolone	38	20	48 h	No effect on cell viability, proliferation inhibited after day 3	Yoon et al. (2010)
Human mesenchymal cells	^{111}In-oxine	10	20	48 h (viability), 14 days (activity)	No effect on viability, decrease in metabolic activity and migration	Gildehaus et al. (2011)

ES embryonic stem

(Bustamante et al. 1997). Various doses of ^{111}In (up to 260 Bq/cell) have been tested in different cell preparations with conflicting data (Table 10.2). Yoon et al. (2010) showed that the viability of rat bone marrow mesenchymal cells labeled with 38 Bq of ^{111}In/cell was not affected, but the cell proliferation was inhibited after the third day of labeling. Jin et al. (2005) showed no loss of cell viability when 0.14 Bq/cell of ^{111}In-tropolone was used, while complete cell death was observed with a dose of 1.2 Bq/cell. Thus, careful dose selection and viability testing are recommended when working with ^{111}In-oxine. An alternative choice is ^{99m}Tc-HMPAO or an approach that labels only a fraction of the cells.

Cell viability seems to be dependent on follow-up time as well (Gholamrezanezhad et al. 2009). In most studies, cell viability was assessed within a few days after labeling (usually 2 days); however, cytotoxicity is evident later. Even a small dose of ^{111}In-oxine resulted in significant cell loss when the follow-up was 2 weeks (Gholamrezanezhad et al. 2009). ^{111}In-oxine labeling (10 Bq/cell) led to moderately impaired metabolic activity and migration of human mesenchymal cells, but viability, stem cell character, and plasticity were preserved during the 2-week follow-up (Gildehaus et al. 2011).

Lastly, different cell types may have different sensitivities to ionization radiation. It is likely that actively dividing cells and those that are not yet fully mature are at the highest risk from the effects of radiation.

10.5 SPECT Imaging for Biodistribution Studies in Stroke

A major question with cell therapies is to know how many of the delivered cells eventually home in on injured tissue. SPECT imaging with different tracers offers an efficient method to study the accumulation of cells in the ischemic brain in relation to other organs over several days after a single injection. However, only a few studies have applied SPECT for cell tracking in stroke animals or patients (Table 10.3).

Mäkinen et al. (2006) studied the biodistribution of ^{111}In-oxine-labeled mononuclear human umbilical cord cells after intravenous infusion in rats with middle cerebral artery occlusion (MCAO) at 30 min and 24 h after administration. Labeled cells ($1–5 \times 10^7$) were infused 24 h after MCAO. The results indicated initial labeled cell accumulation within lung tissue and later relocation to the liver after 24 h, without signal in the ischemic brain. A similar distribution pattern was observed with ^{99m}Tc-HMPAO-labeled human mesenchymal stem cells in MCAO rats (Detante et al. 2009) and with other cell preparations in naïve rats (Gao et al. 2001), in rats with myocardial infarction (Barbash et al. 2003), and after brain trauma in rats (Park et al. 2011). The heavy entrapment of cells to the lungs limits efficient homing to the target tissue, and it may at worst lead to respiratory failure (Fig 10.1). The use of vasodilators has been suggested in order to minimize lung adhesion (Gao et al. 2001).

Intra-arterial cell infusion is an alternative route to circumvent trapping in the internal organs and to target cells toward the ischemic brain (Lappalainen et al.

Table 10.3 SPECT studies in experimental stroke animals and patients

Cell type	Model/patient characteristics	Administration route, time	Cell tracking, time	Distribution pattern	Comments	Reference
Human UCB cells	Rat tMCAO	Femoral vein, 24 h after ischemia	SPECT/CT, ^{111}In-oxine, immediately and 24 h	Lung > liver > spleen (immediately)	Relocation from lung to internal organs, no signal in brain	Mäkinen et al. (2006)
Autologous mononuclear bone marrow cells	Ischemic stroke in the left MCA territory	Left MCA, 9 days after stroke	SPECT, ^{99m}Tc-HMPAO, 8 h	Left brain = bladder > liver > spleen (8 h)	A case report, strong signal in ischemic brain	Correa et al. (2007)
Human ES cells, rat hippocampal cells	Rat tMCAO	Common carotid artery or femoral vein, 24 h after ischemia	SPECT/CT, ^{111}In-oxine, immediately and 24 h	Liver > spleen > kidney (24 h)	Minor signal in ischemic brain after intra-arterial administration	Lappalainen et al. (2008)
Human mesenchymal stem cells	Rat tMCAO	Saphenous vein, 7 days after ischemia	Gamma-camera, ^{99m}Tc-HMPAO, 2 and 20 h	Lung > kidney > liver > spleen	Relocation from lung to internal organs	Detante et al. (2009)
Autologous bone marrow mononuclear cells	Cerebral infarct in the MCA territory	Intra-arterial, 59–82 days after stroke	SPECT, ^{99m}Tc, 2 and 24 h	Liver > bladder > lungs > kidney (2 h)	Accumulation at 2 h in ischemic hemisphere, major uptake in liver	Barbosa da Fonseca et al. (2010)

ES embryonic stem, *MCA* middle cerebral artery, *tMCAO* transient middle cerebral artery occlusion, *SPECT* single photon emission computed tomography, *UCB* umbilical cord blood

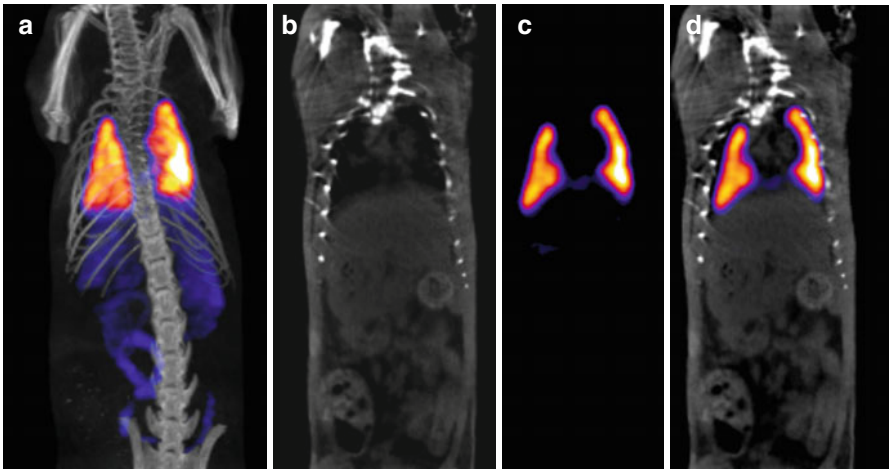

Fig. 10.1 Intravenous cell delivery. The majority of ^{99m}Tc-HMPAO-labeled human bone marrow-derived mesenchymal cells are located in the lungs 30 min after intravenous infusion. A maximum intensity projection SPECT/CT fusion image (**a**) and individual coronal image slices of CT (**b**), SPECT (**c**), and co-registered SPECT/CT data (**d**) are shown. Images were obtained with a dedicated small animal scanner NanoSPECT/CT (Bioscan Inc, Washington DC, USA). SPECT is presented in colors, and CT is shown as an anatomical reference in grayscale (Courtesy of the Finnish Red Cross Blood Service and the Centre for Drug Research, University of Helsinki, Finland).

2008; Walczak et al. 2008; Li et al. 2010; Chua et al. 2011). Indeed, intra-arterial infusion of human embryonic stem (ES) cell-derived neural progenitors (1×10^6) resulted in minor engraftment into the ischemic hemisphere (<1 %), while no SPECT signal was detected after intravenous infusion (Lappalainen et al. 2008). As a complication, the intra-arterial cell injection may be associated with high mortality, possibly due to microvascular occlusions (Walczak et al. 2008; Li et al. 2010). It was recently shown that this may partly be due to injection technique (Chua et al. 2011). To support this notion, infusion of human bone marrow-derived mesenchymal cells directly into the external carotid artery showed immediate cell entrapment in the brain without any complications (Fig 10.2). However, most of the cells or signal again relocated to the internal organs during the following 24 h (Mitkari et al. 2012).

SPECT imaging is also truly translational, and the same tracers can be used in patients (Correa et al. 2007; Barbosa da Fonseca et al. 2010). Barbosa da Fonseca et al. (2010) labeled autologous bone marrow mononuclear cells (2×10^7) with ^{99m}Tc and followed the distribution of the cells after intra-arterial delivery in six patients at 2 and 3 months after stroke. They were able to show that the cells remained at the site of the lesion for 2 h while the remaining uptake was mainly distributed to the liver, lungs, spleen, kidneys, and bladder. It is unclear whether this short time of location will be enough for any therapeutic benefit. A recent review by McColgan et al. (2011) describes the range of other methods employed in cell tracking in patients (e.g., PET, MRI).

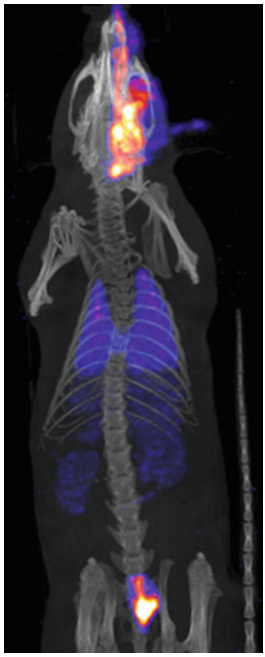

Fig. 10.2 Intra-arterial cell delivery. ^{99m}Tc-HMPAO-labeled human bone marrow-derived mesenchymal cells were infused into the external carotid artery 24 h after middle cerebral artery occlusion (MCAO) in rats. The majority of cells were located in the ischemic hemisphere 30 min after infusion (Courtesy of the Finnish Red Cross Blood Service and the Centre for Drug Research, University of Helsinki, Finland).

Conclusions

SPECT is a noninvasive imaging modality that allows repeated imaging and quantitative cell tracking both in stroke animals and patients. Imaging using dedicated small animal SPECT scanners has revealed the limitations of the use of intravenous cell infusions (e.g., acute massive lung entrapment), which can be partly circumvented by intra-arterial delivery. More importantly, experimental results have been confirmed in stroke patients, which strongly support the translational role for SPECT in assisting the appropriate choice of cell type, delivery route, and dosing for effective and safe therapy.

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Part III

Early Phase Clinical Studies

Clinical Trials: Intracerebral Cell Therapy in Stroke Patients

11

Douglas Kondziolka, Gillian Harrison, and Lawrence Wechsler

11.1 Introduction

The use of stem cell therapy for stroke is a burgeoning area of clinical research. Buoyed by promising studies performed in preclinical animal models of stroke, in which transplanted cells resulted in functional improvement (Borlongan et al. 1998; Chen et al. 2001a, b; Chopp and Li 2002; Guzman et al. 2008; Hicks et al. 2009; Stroemer et al. 2008), the study of intracerebral cell therapy for stroke has progressed to early phase clinical trials.

Though it is clear that transplanted cells convey a functional benefit, the mechanism by which this occurs is not fully known. Transplanted cells are hypothesized to provide benefit not only directly through cell replacement of damaged tissue but also by providing trophic, neuroprotective, and immunomodulatory support. Lack of clear mechanism is but one of the challenging considerations encountered in the design of clinical trials. Particular concerns that may impact the potential success of cell therapy include the anatomy, vascular supply, timing of stroke, site and technical

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delivery of cell implantation, target patient population, and selection of appropriate outcome measures (Locatelli et al. 2009; Savitz et al. 2004).

A variety of cell types have been investigated in experimental models and translated to clinical trials. Published trials of intracerebral cell therapy studied cells derived from embryonic carcinoma lines (Kondziolka et al. 2000, 2005), fetal porcine striatum (Savitz et al. 2005), and bone marrow (Suárez-Monteagudo et al. 2009). More recently, investigators around the world have continued to evaluate the use of both autologous marrow-derived (NCT00950521) and allogeneic cord blood-derived (NCT01438593) cells, as well as neural stem cells (NCT01151124). Current studies in the United States have focused on the use of modified marrow stromal cells (NCT01287936). This review summarizes the four published clinical trials of intracerebral cell therapy for stroke and describes the ongoing study of such therapy in the United States (Table 11.1).

11.2 Preclinical Basis for Clinical Trials

Several investigators have evaluated the use of transplanted fetal tissue, rat striatum, or cellular implants in small animal stroke models (Johansson and Grabowski 1994; Kleppner et al. 1995). Although transplanting primary human fetal neurons into patients with neurodegenerative disease may exhibit favorable preclinical results, the widespread use of such cells is likely to be limited due to ethical and logistic difficulties inherent in obtaining large quantities of fetal neurons (Thompson et al.

Table 11.1 Overview of published and ongoing clinical trials of intracerebral stem cell therapy for stroke

Reference	Cell type	Study design	Number of patients	Outcome	Trial status
Kondziolka et al. (2000)	NT2	PI, R-SB	12	Safe and feasible	Complete
Kondziolka et al. (2005)	NT2	PII, R-SB	18	Safe and feasible. No significant benefit in motor function	Complete
Savitz et al. (2005)	Fetal porcine LGE	PI, NR	5	Terminated by FDA after AEs in two patients	Complete
Suárez-Monteagudo et al. (2009)	Autologous bone marrow-derived mesenchymal stem cells	PI, NR	5	Safe and feasible	Complete
NCT01287936	Bone marrow stromal cells	PI/IIA, NR	18	–	Ongoing

Abbreviations: *NT2N* NTERA-2 human embryonic carcinoma-derived cell line, *PI* phase I, *PII* phase II, *R* randomized, *SB* single blind, *LGE* lateral ganglion eminence, *FDA* food and drug administration, *AE* adverse event

1999); thus, much effort has been devoted to developing alternate sources of cells for use in transplantation.

The first of such sources to be translated into the clinical realm was the NTERA-2 cl.D1 (NT2) human embryonic carcinoma-derived cell line. These cells were shown to proliferate in culture and further differentiate into pure, postmitotic human neuronal cells (LBS-Neurons) upon treatment with retinoic acid (RetA) (Andrews et al. 1984; Pleasure and Lee 1993). During induction, the LBS-Neuronal precursor cells undergo significant changes resulting in the loss of neuroepithelial markers and the appearance of neuronal markers. The final product is a >95 % pure population of human neuronal cells that are virtually indistinguishable from terminally differentiated, postmitotic neurons (Andrews et al. 1984; Pleasure and Lee 1993); accordingly, they appear to function as central nervous system (CNS) progenitor cells with the capacity to develop mature neuronal phenotypes. When transplanted into mouse models, NT2 cells survived, extended processes, expressed neurotransmitters, formed functional synapses, and integrated into the host tissue (Kleppner et al. 1995; Trojanowski et al. 1997).

Preclinical studies of LBS-Neurons were carried out in rat models of transient focal, rather than global, ischemia to maximize the chance of functional recovery. In several studies, animals received ischemic insults localized to the striatum. Animals that displayed significant behavioral deficits 1-month post-insult received cell transplantation with LBS-Neurons and cyclosporine A (CsA) treatment. Over the 6-month observation period, animals displayed amelioration of ischemia-induced deficits, including complete recovery in the passive avoidance test and normalization of motor function in the elevated body swing test. These benefits contrasted with control groups of rats receiving fetal cerebellar cells, medium alone, or CsA alone, which failed to show behavioral improvement (Borlongan and Sanberg 1995; Borlongan et al. 1997; Saporta et al. 1999). Subsequent studies demonstrated graft survival with mature neuronal phenotypes and integration into the host brain (Kleppner et al. 1995; Trojanowski et al. 1993, 1997). Viable cells were demonstrated in 90 % of recipients, with graft survival observed up to 14 months posttransplant, and electrophysiologically tested differentiation into fully mature neuronal phenotypes (Borlongan and Sanberg 1995; Kleppner et al. 1995).

A second alternate source of cells investigated for neurotransplantation was porcine primordial striatum, also known as the lateral ganglion eminence (LGE). Cells harvested from the LGE are known to develop into striatal GABAergic projection neurons and were first evaluated in animal models of Huntington's disease; graft survival and host integration, as well as improvement of functional neurologic deficit were demonstrated (Deacon et al. 1994; Isacson et al. 1995; Pakzaban et al. 1993). In these early studies, animals followed for up to 15 months were found to have axons and glial fibers with maturational changes typical of pig striatum (Isacson et al. 1995). Studies of immunosuppressed rats with lesions of the corpus striatum analyzed grafts for development with respect to donor age, cell dosage, and survival up to 22 weeks postimplant. Prolonged development of striatal cells was observed, with long-distance, target-specific axonal growth into the host brain (Deacon et al. 1994). Later studies of LGE cells in rat models of middle cerebral artery (MCA)

occlusion showed that cells transplanted to ischemic striatum 3–28 days after stroke led to implant survival, with solid grafts observed to fill the infarct cavity; cells differentiated into glia and neurons, elaborated extensive processes into the host brain, showed evidence of synaptogenesis, produced neurotransmitters, and expressed typical neuronal proteins. Fourteen days post-stroke, these animals showed significant functional improvement compared to controls (Dinsmore et al. 2002).

Cells derived from bone marrow have also been investigated for use in cell therapy for stroke patients. In addition to hematopoietic stem cells, bone marrow contains mesenchymal stromal cells and a portion of multipotent cells that differentiate into tissues of mesenchymal lineage, such as osteoblasts, chondroblasts, adipocytes, and skeletal muscle (Tang et al. 2007). Two studies of CD34+ cells harvested from bone marrow and given either systemically or incracranially to animal models of stroke demonstrated evidence of functional recovery and reduced infarct size (Shyu et al. 2006; Taguchi et al. 2004). Further studies of rat MCA occlusion models have demonstrated improved recovery with mesenchymal cells administered in a variety of manners and beginning 1-day post-infarct. Delayed delivery, even as far as 1-month post-infarct, continued to convey long-term functional improvement (Shen et al. 2007). These studies were translated into successful clinical trials of IV mesenchymal stem cells; treatment in both the acute and subacute post-stroke period was found to be safe and feasible on short- and long-term follow-up (Bang et al. 2005; Honmou et al. 2011; Lee et al. 2010; Savitz et al. 2011b). Intracerebral delivery, however, results in more transplanted cells in the brain directly targeting the lesion when compared to systemic modes of administration (Jin et al. 2005). Though the underlying mechanism remains unclear, given that only a small percentage of cells have been observed to survive for long periods near the ischemic region or express neuronal markers, it is less likely that they exert benefit by replacing cells (Coyne et al. 2006) and perhaps more probable that they enhance functional outcomes by indirectly supporting repair mechanisms (Luo 2011) through trophic factors or specific cell types, such as the CD34+ subpopulation (England et al. 2012).

One recently developed cell line utilizing bone marrow is SB623 (SanBio, Inc., Mountain View, CA), which is a line of human bone marrow-derived stromal cells transiently transfected with a plasmid encoding the intracellular domain of Notch-1 (Dezawa et al. 2004), a human heterodimeric transmembrane receptor important for transcription activation. In vivo rat models of MCA occlusion have been treated with CsA and escalating doses of intracranial SB623 at 1-month post-infarct. As early as 7 days posttransplant, these animals showed significant behavioral improvement on both elevated body swing test and Bederson score, with a trend toward a dose response with escalating numbers of cells (Yasuhara et al. 2009). Interestingly, only 7–9 % cell survival, with less than 1 % neural differentiation, was observed, supporting a trophic mechanism of action. Later studies with 3- and 6-month follow-up showed similar recovery of motor and neurologic function, with continued improvement over time, as well as the ability to transplant cells safely without the use of CsA (Yasuhara et al. 2009).

11.3 Clinical Trial Design

Thus far, only early phase clinical trials with small patient populations have been performed. Trials recruited fairly diverse study populations; however, study participants were all adults >18 years old with "stable" motor deficit (unchanged over a period of time) from "chronic" stroke occurring from 3 months up to a maximum of 10 years prior to baseline. All studies excluded patients with severe or uncontrolled chronic diseases, malignancies, or disabling psychiatric conditions.

LBS-Neurons were the first human cells to be tested in clinical trials. A phase I open-label single-blind study was performed with primary safety and secondary efficacy outcomes. Twelve patients with basal ganglia stroke 6 months to 6 years prior to transplant and stable motor deficit were recruited. Two cohorts were examined—the first comprised of four patients receiving two million cells and the second comprised of eight patients randomized to either two or six million cells. Patients were followed for 52 weeks, and the primary outcomes were analyzed at 24 weeks postoperatively (Kondziolka et al. 2000).

A subsequent phase II open-label single-blind trial was performed. Potential subjects must have been age 18–75 years and experienced an ischemic or hemorrhagic infarction involving the basal ganglia 1–6 years prior to enrollment with no substantial change in neurological deficit for ≥2 months. Two cohorts of nine patients each were randomized to receive either cells plus rehabilitation or rehabilitation alone. In the first cohort, patients received five million cells plus rehabilitation ($n=7$) or rehabilitation alone ($n=2$). In the second cohort, patients received ten million cells and rehabilitation ($n=7$) or rehabilitation alone ($n=2$). Patients were followed at predetermined intervals for 52 weeks, with primary outcome for statistical analyses examined at 6 months. Studies evaluated safety of transplantation using neurologic exams, serial MRI and positron emission tomography (PET), laboratory tests, and documentation of all adverse events. The phase I trial used the National Institutes of Health Stroke Scale (NIHSS), European Stroke Scale (ESS), Barthel Index (BI), and SF-36 Health Survey to evaluate functional disability and quality of life. In addition to these assessments, the phase II trial also included use of the Stroke Impact Scale (SIS), Fugl-Meyer Assessment of Motor Recovery After Stroke, gait tests, Action Research Arm Test, and Grooved Pegboard Tests (Kondziolka et al. 2000, 2005).

Similarly, Savitz et al. (2005) performed an open-label trial with intentions to recruit 12 subjects. Inclusion criteria varied somewhat; however, patients were limited to only those with ischemic stroke from 3 months to 10 years prior to baseline with an MCA infarct of 20–100 cm^3 and affecting the striatum, resulting in permanent neurologic deficits and moderate disability. Patients were followed serially for safety and efficacy up to 24 months. Evaluations included physical examinations, NIHSS, Modified Rankin Scale (mRS), BI, MRI, and laboratory tests, which notably included polymerase chain reaction for porcine endogenous retrovirus.

For autologous bone marrow transplant, a small, open-label trial of five patients was planned. Participants were required to be between 40 and 70 years old with disabling motor sequelae from stroke occurring between 1 and 10 years prior to

enrollment. Investigators studied a comprehensive battery of safety and efficacy outcomes. Neurological status was assessed using NIHSS, Scandinavian Stroke Scale (SSS), BI, and SF-36 in addition to scales evaluating spasticity, gait, and equilibrium. Neurocognitive status was assessed using the Mini-Mental Status Examination (MMSE); Wechsler Adult Intelligence Scale (WAIS); Rey memory, learning, and complex figures tests; and a selection of additional attention, memory, language, frontal executive function, and depression scales. Neurophysiologic and radiologic assessment consisted of electroencephalography (EEG), SPECT, MR spectroscopy, and transcranial magnetic stimulation (TMS). Patients were assessed at baseline, 6 and 12 months postoperatively.

The ongoing trial of SB623 cells (NCT01287936) being conducted at the University of Pittsburgh and Stanford University is an open-label phase I/IIA with primary safety and secondary efficacy outcomes. Eighteen patients between 18 and 75 years of age with ischemic stroke in the MCA territory occurring 6 months to 3 years prior to baseline will be recruited. Participants must have "stable" neurologic deficits of NIHSS > 7 and mRS of 3–4 with no change during the 3 weeks prior to enrollment. Three dose-escalation cohorts of six patients, each receiving 2.5, 5, or 10 million cells, will be assessed. Similar to prior trials, safety outcomes evaluated will include record of adverse events, physical examinations, MRI, and laboratory tests for 24 months postoperatively. Efficacy parameters to be assessed include NIHSS, ESS, mRS, Fugl-Meyer, changes on PET scan, and a comprehensive neurocognitive battery.

11.4 Cell Preparation and Surgical Method

Cell preparation prior to the day of surgery was cell and source specific. All investigators used stereotactic surgical procedures for intracerebral injection of cells with either computed tomography (CT) or magnetic resonance image (MRI) planning.

LBS-Neurons (Layton BioScience, Inc., Gilroy, CA) were produced using antibiotic-free conditions in a class 10,000 clean room according to cGMP protocols. The NT2/D1 human precursor cell line was plated in culture from a well-characterized working cell bank. This stock culture was passaged twice per week in DMEM/F-12 growth media. NT2/D1 cells were then induced to differentiate with 10 μM RetA. After 6 weeks of treatment, cultures were harvested with trypsin/EDTA and replated at lower cell densities, then maintained in DMEM/F12 media containing 5 % FBS and a mitotic inhibitor mixture for 6 days. Cells were selectively harvested, purified, and extensively tested, then cryopreserved in freezing media and stored in the vapor phase of liquid nitrogen. On the day of surgery, 1 h prior to implantation, vials were thawed, gently washed twice with Isolyte S (McGaw, Inc., Irvine, CA) and centrifuged at 200 × g for 7 min at room temperature, then the cell pellet was resuspended in Isolyte S. Viable cell count was determined with a sample of LBS-Neuron suspension using 0.4 % trypan blue, and the cells were resuspended to a concentration of 3.3×10^7 cells/mL in Isolyte S and aliquoted at 120 μL/sterile 1.0 mL vial. An aliquot was considered acceptable if greater than 50 % of cells were

viable and cells were immediately transferred to the operating room (OR) upon completion of preparation (Kondziolka et al. 2005).

A stereotactic surgical procedure was used for intracranial injection of cells. CT was used for operative planning of safe trajectories that entered a cortical gyrus and spared a sulcus. In the phase I trial, the first four patients received a single-pass injection of two million cells divided into three implants of 20 μL each. On a brain weight basis, this was 1/20th of the effective dose in rats. The subsequent eight patients received either a single, two million cell pass or three-pass injections of six million cells in nine implants (Kondziolka et al. 2000). In the phase II trial, five cell implants, spaced equally across a distance of 20–25 mm, along five trajectories, for a total of 25 deposits, were planned. One point in the basal ganglia inferior to the center of the stroke and four other targets anterior, posterior, medial, and lateral to the central target, spaced by 5 mm, were chosen (Kondziolka et al. 2004). A stabilizing probe, 1.8 mm in outer diameter and 15 cm long, was inserted first to a point 4 cm proximal to the final target. Cells were injected at a rate of 5 μL/min, for a total implantation time of approximately 150 min (Kondziolka et al. 2005).

When studying autologous bone marrow neurotransplantation, Suárez-Monteagudo et al. (2009) required patient donation on the day prior to surgery. Patients were taken to the OR, and samples of 120–150 mL of bone marrow were aspirated from the posterior iliac crest, collected in sterile plastic bags containing CDP-adenine, and transported to the cell culture lab for processing. Mononuclear cells were isolated by a Ficoll-Hypaque density gradient and resuspended in heparinized (10,000 UI/L) saline, according to institutional practice (Hernandez et al. 2007). A 40–60 mL concentrated cell suspension was obtained, washed twice with saline, and centrifuged at 1,200 × g for 10 min at 4 °C. DNAse (0.003 %) was then added to the cell suspension and centrifuged at 650 × g for 5 min. The pellet was resuspended in an appropriate volume of saline and stored at 4 °C until transplant 24 h later. A small fraction was used for cell counting, viability testing by trypan blue exclusion, and microbiological tests. CD34+ cell analysis was carried out using fluorescence-activated cell sorting.

Surgical planning was performed using CT images and STASSIS software (CIREN, La Habana, Cuba). The final implant location was based on combined information from single-photon emission tomography (SPECT) to quantify blood flow, 1.5 T MRI for high anatomic resolution, and consideration of accessibility, size, topography, and morphology of the lesion. Recordings of multi-unit electrical activity were performed transoperatively using a deep recording system (NDRS, CIREN, La Habana, Cuba) to confirm existence of neuronal activity in the perilesional area. Cells were implanted using several tracts around the target, using a Rehncrona canulla and eight deposits of 2.5 μL per tract (Suárez-Monteagudo et al. 2009).

For surgical implantation of SB623 cells, 1 mL sterile suspensions containing 5×10^6 cells/mL cryopreserved in CRYOSTORE™ freezing media are provided by SanBio within 2 weeks of the surgical date. On the morning of surgery, the preserved cells are thawed, washed, centrifuged, and resuspended in Plasma-Lyte A at the necessary concentration for administration to the patient within 3 h of resuspension.

After applying a standard stereotactic frame (Leksell Stereotactic System, Elekta), 1.5 T MRI is used for surgical planning, identifying a trajectory that enters a gyrus and spares a sulcus. Targets are identified in the basal ganglia inferior to and above the motor region of the stroke. Five cell deposits, spaced 4–5 mm apart with 2–3 implants within the penumbra distal to the stroke area and 2–3 implants within the penumbra proximal to the stroke area, along three trajectories, for a total of 15 deposits, are delivered. Accessible target locations closest to the motor pathways are selected. After creating one 1–1.5 cm burr hole and opening the dura, a long stabilizing cannula (1.8 mm outer diameter, 15 cm long) with a removable solid stylet is inserted to a point just proximal to the penumbra of the stroke area. Following stylet removal, a Pittsburgh Implantation Cannula (0.9 mm outer diameter, 19 cm long, 20 μL total volume) is inserted to the deepest target point. Cells are injected at a rate of 10 μL/min and 20 μL/deposit, for a total deposit of 100 μL/tract and implantation time of approximately 60 min, with a maximum allowable time of 3 h from cell preparation to final implantation (NCT01287936).

11.5 Need for Immunosuppression

The decision to immunosuppress patients undergoing neurotransplantation may be based on a number of factors, including the source of graft, type of cell product, and consideration of any immunomodulatory effects the cells may have (Savitz et al. 2011a). Patients participating in trials of LBS-Neurons were treated with oral CsA, 6 mg/kg/day for 1 week prior to surgery and 8 weeks postoperatively during the phase I trial (Kondziolka et al. 2000) and 6 mg/kg twice daily 1 week prior to surgery and for 6 months postoperatively in the phase II trial (Kondziolka et al. 2005). Patients also received intraoperative methylprednisolone (40 mg IV) during both studies.

On the other hand, no other clinical trials have required immunosuppression of study participants. Xenografts of LGE cells were treated with anti-MHC class I $F(ab')_2$ fragments lacking the Fc region (PT85, Veterinary Medicine Research and Development, Inc.) prior to transplantation. Graft survival has been shown to persist using this immunosuppressive technique after intracerebral porcine cell transplantation in animal models of Huntington's and Parkinson's disease and stroke (Dinsmore et al. 2002; Pakzaban et al. 1995).

Further, studies of autologous bone marrow transplant would not require immunosuppression given the etiology of cells. Lastly, preclinical studies of rats treated with SB623 cells with and without CsA showed no significant differences in improvement; compared to controls, rats receiving SB623 still exhibited significant benefit, even without treatment with CsA. Additionally, histologic evaluation of experimental and control groups displayed no eventful inflammation or immune response and no obvious difference between the two specimens. Markers of immunoreactivity were comparable across all treatment groups. The lack of differences in inflammatory and immune markers provides evidence that intracerebral transplantation of SB623 does not elicit overt host reactions (Yasuhara et al. 2009).

11.6 Results

11.6.1 Patient Population

Patient demographics and clinical characteristics for each of the completed trials are described in Table 11.2. The phase I trial of LBS-Neurons recruited 12 patients (9 men, 3 females) age 44–74 years old with a mixture of cardiac, renal, endocrine, and psychiatric comorbidities. There were no significant differences between cohorts for age, height, and weight. Mean time since onset of infarct was 27 months (range 7–55), with stroke involvement limited to the basal ganglia in eight patients and including an additional extensive region of cortex in four patients. Comparatively, the phase II trial recruited 18 patients (13 men, 5 women) age 24–70 years old with a similar mixture of comorbidities. Mean time since onset of stroke was 3.5 years (range 1–5); nine were ischemic and nine were hemorrhagic.

Due to adverse events, the study of LGE cells was stopped early after enrollment of five patients. Of those treated, three were men and two were women. Patients were aged 25–52, and mean time since onset of stroke was 5 years (range 1.5–10). All patients had MCA strokes with contralateral hemiparesis, and a mixture of right- and left-sided infarcts was included. Comorbidities were minimal and included hyperlipidemia, diabetes mellitus, partial seizure (one patient), use of a baclofen pump (one patient), and factor V Leiden.

Investigators studying autologous bone marrow transplant recruited a total of five patients, as well; participants were three men and two women, aged 41–64 years old, who were 3–8 years post-stroke. Both ischemic and hemorrhagic strokes were included and were located in the thalamus, striatum, or primary motor cortex.

11.6.2 Cell Viability

Cell viability was only minimally reported in published clinical trials. Kondziolka et al. (2000, 2005) required >50 % viability for administration, and Savitz et al.

Table 11.2 Patient demographic and clinical characteristics

Reference	Mean age, years (range)	Females (F), males (M), count	Type of stroke, count	Mean time since onset, years (range)
Kondziolka et al. (2000)	61 (44–74)	3 F, 9 M	12 ischemic	2.25 (0.58–4.8)
Kondziolka et al. (2005)	59, 5 M 58, 10 M 46 control (24–70)	5 F, 18 M	9 ischemic, 9 hemorrhagic	3.5 (1–5)
Savitz et al. (2005)	40 (25–52)	2 F, 3 M	5 ischemic	5 (1.5–10)
Suárez-Monteagudo et al. (2009)	51 (41–64)	2 F, 3 M	3 ischemic, 2 hemorrhagic	5 (3–8)

(2005) required >70 %; however, individual patient viability data was not reported. This may reflect the controlled, highly predictable, and reproducible nature of laboratory preparation of cells prior to administration. In contrast, studies of autologous bone marrow neurotransplantation utilized participant donation on the day prior to surgery; thus, quality and viability of cell samples were much more unpredictable compared to trials of other cell types. To this end, Suárez-Monteagudo et al. (2009) reported cell implant viability operative data. Preoperatively, injected cell volume ranged from 115 to 220 μL with 76–94 % viability. Total number of injected cells ranged from 14 to 55×10^6 cells; notably, total number of transplanted cells and %CD34+ cells were not directly correlative with injected volume. Postoperatively, viability ranged from 50 to 92 %. Patients received 46–88 deposits of cells distributed in 6–15 tracts.

11.6.3 Safety

Implantation of LBS-Neurons was deemed safe and feasible upon completion of phase I and phase II clinical trials. Implantation was successfully performed in all 26 patients with no evidence of hemorrhage or new neurological deficit identified in the immediate postoperative period. Two new neurological events did occur; however, one patient experienced a single seizure the day after implantation and one patient, who was on aspirin and ticlopidine after surgery, was found to have a chronic subdural hematoma requiring surgical drainage 1 month after surgery. A small risk for both of these events should be expected, given the cortical transgression, spinal fluid loss, and minor accumulation of air in the brain during the procedure that may increase risk of seizure and the frequency with which stroke patients are managed with antiplatelet agents or other anticoagulants conveying some risk of delayed intracranial hemorrhage. In the long term, no adverse events related to implantation occurred at 24–36 months follow-up for phase II patients nor did phase I patients experience any adverse events at 52–60 months after implant (Kondziolka et al. 2005).

Unfortunately, use of LGE cells was associated with complications in some subjects. Though there were no perioperative complications and the first 3 patients did not experience adverse events during the 4 years following implantation, the fourth patient (with history of right MCA infarct) developed progressive left arm and leg weakness at postoperative day 20. MRI showed an area of enhancement in the right frontal lobe remote from the area of infarct and surgical implant site. Subsequent biopsy showed bland necrosis with macrophages and T cells, as well as adjacent areas of organization suggestive of infarct; it was negative for pig repetitive DNA element. The patient returned to baseline after 10 days and a course of steroids. An independent study board concluded that he experienced a cortical vein occlusion, likely secondary to the surgical procedure. The right frontal lobe enhancement resolved after 5 months, and after a complex partial seizure when the patient decreased his gabapentin at 6 months, he had no further adverse outcomes. Patient 5 also experienced an adverse outcome consisting of generalized and partial complex seizures while hyperglycemic (glucose up to 630). MRI showed a ring-enhancing

lesion in the right frontal lobe, subadjacent to the site of the first burr hole, and an area of mild enhancement within the infarct. The ring-enhancing lesion resolved within 3 months and the mild enhancement remained unchanged on follow-up imaging. On long-term follow-up, the patient experienced no further clinical events related to cell implantation. Following the adverse events experienced by patients 4 and 5, the study of LGE cells was terminated by the Food and Drug Administration (FDA) (Savitz et al. 2005).

Patients who received autologous bone marrow cell therapy did not experience any clinically significant adverse events related to the surgical procedure or cell implantation. Investigators classified health events in the 90-day postoperative period as "surely related," "probably related," "less probably related," and "not related." The only "surely related" or "probably related" events were headache, drowsiness, nausea, and hyperglycemia, which all resolved within 24–48 h after the surgery. In the long term (90 days–1 year), no adverse events related to the surgery or cells were observed (Suárez-Monteagudo et al. 2009).

11.6.4 Functional Outcomes

All studies used combinations of validated tools for assessment of neurologic deficit and level of function in stroke patients; number and type of outcome measures were varied, as were time points and length of follow-up for assessment of efficacy of therapy.

Studies of LBS-Neurons used ESS as the primary evaluation of functional improvement, which is validated for changes ±3 points. For the phase I study, baseline mean ESS was 60.1 for the two million cell group and 61.3 for the six million cell group. At the 24-week follow-up, 6 of 12 patients had improved ESS (range 3–10 points), 3 patients were unchanged, and 3 patients deteriorated (range −1 to −3 points). Mean change from baseline to 24 weeks for all patients was 2.9 points ($p = 0.046$), and there was no correlation between clinical outcome and size of infarct or time since infarction. No statistically significant change was seen in NIHSS, BI, or SF-36 at 24 weeks, though a trend toward improvement was observed for NIHSS. At 2 years, the mean ESS change was −1.4 in the two million cell group and +8.5 in the six million cell group ($p = 0.05$).

For the phase II study, ESS was once again used as the primary functional outcome; scores recorded at weeks 24, 26, and 28 were averaged to account for variations in patient effort at any one particular visit. Of the 7 patients receiving 5 million cells, 4 had improved ESS scores (range 5.3–15 points), 2 were unchanged, and 1 was decreased (−4.5 points) compared to baseline. Comparably, in the ten million cell group, 2 of 7 patients had improved scores (6.5 and 14.5 points), 2 were unchanged, and 3 had decreased scores (−4.5 to −5.5 points). Of the controls, 1 of 4 patients had an improved score (3.5 points) and the other 3 remained unchanged. For all patients receiving LBS-Neurons, mean change in ESS from baseline to 6 months was 2.7 points, compared to a mean change of 0.75 points in controls ($p = 0.148$). When comparing mean 6-month ESS scores to mean baseline scores of

treated patients, the observed change of 4.74 points was not significant ($p=0.146$). No difference in NIHSS was observed when comparing all patients who received cells to controls or when comparing the five and ten million cell groups. The SIS, a measure of degree of disability caused by stroke and the effects of surgery, was higher (higher daily activity) in treated patients compared to controls ($p=0.056$), though significant change was only noted when comparing scores for patients with implants at 6 months and baseline ($p=0.045$). Everyday memory scores in patients with implanted cells were also significantly improved compared to controls ($p=0.012$) and when comparing 6-month scores with the patients' own baseline scores ($p=0.004$). Though no significant change in Fugl-Meyer scores was noted when patients receiving LBS-Neurons were compared with controls, a trend toward improvement in hand movement (mean change 1.15 points [95 % CI 0.07–2.4], $p=0.06$) and wrist movement (mean change 0.92 [95 % CI 0.05–1.9], $p=0.06$) was observed. Authors also evaluated grasp, grip, pinch, and gross movement using the Action Research Arm Test. At 6 months, treated patients had significantly improved gross-movement scores compared to controls. Overall score in treated patients compared to their own baseline was significant in both the five million cell group ($p=0.043$) and ten million cell group ($p=0.051$). Significant changes were observed in gross movement and grasp but not grip and pinch movements.

When investigating use of LGE cells, Savitz et al. (2005) used NIHSS, mRS, and BI. Anecdotally, authors reported that several patients experienced improvement in baseline aphasia, motor strength, ambulation, and spasticity; however, only one patient had significant change in NIHSS (>4 points), comprised of speech improvement from aphasia/dysarthria (incomprehensible speech) to fluent speech with only very occasional word finding difficulty, as well as mild improvement in right arm strength. All reported changes were sustained after 4 years of follow-up. No significant changes in functional outcome as measured by mRS or BI were reported nor did any patient experience deterioration compared to baseline.

Using an extensive battery of functional outcome measures, authors investigating autologous bone marrow cell therapy reported statistically significant functional improvement. Compared to baseline, patients showed improved motor neurologic condition based on both NIHSS and SSS at 12 months ($p<0.05$). They also showed reduced spasticity as measured by the Ashworth scale ($p<0.05$) and increased functional capacity assessed using BI ($p<0.05$). Equilibrium and locomotion were also significantly improved as early as 6 months postoperatively. Though no significant change in SF-36 was reported, all five patients did report improvement of the item concerning limitations due to the physical condition (Suárez-Monteagudo et al. 2009).

11.6.5 Neuropsychological Outcomes

Neuropsychological testing was included in the phase II study of LBS-Neurons and in the study of autologous bone marrow transplant. Both studies aimed to assess similar measures of verbal intelligence, mood, and five domains of cognitive

function (language, attention, learning and memory, visuospatial/constructional ability, mental flexibility). Testing was done at baseline and at 6 months following LBS-Neuron implantation and included the New Adult Reading Test, Controlled Word Association Test, subtests from the Wechsler Memory Scale III, Rey Complex Figure Test, Rey Auditory Verbal Learning Test, Block Design from the WAIS, Beck Depression (BDI-2), and Beck Anxiety Inventories (BAI). There was no evidence of depression in any patient, either at baseline or 6 months. Few changes were associated with treatment on any neurocognitive test, with the exception of one; significant improvements were evident on the Rey Complex Figure Test, which assessed visuospatial/constructional ability and nonverbal memory sensitive to lesions in the nondominant hemisphere. The four patients who demonstrated the greatest improvement in this area all had strokes in the nondominant hemisphere; all of these patients employed a more organized approach and demonstrated improved image reproduction on both immediate and delayed recall. Overall, patients who have strokes in the nondominant hemisphere tended to demonstrate greater improvement in visuospatial/constructional skills and in nonverval memory compared to those who had strokes in the dominant hemisphere; for example, in nondominant stroke subjects, the difference in improvement on the Rey Figure Test Immediate Recall exceeded two standard deviations compared to dominant stroke subjects (Stilley et al. 2004).

Authors reported the results of neuropsychological testing in patients receiving autologous bone marrow cell therapy as either improved, no change, or worsened, with differences defined as postoperative change greater than one standard deviation when compared to the patient's own baseline for each measure. Summaries of intellect, attention, verbal memory, nonverbal memory, language function, frontal/executive function, and depression were reported. Results were mixed and difficult to interpret. One patient showed a trend to worsen in several areas, with the exception of noted improvement in nonverbal memory. The remaining four patients showed a trend to improve in most functions; however, no one specific area was more notable than the others (Suárez-Monteagudo et al. 2009).

11.6.6 Imaging

With the exception of the lesions observed following LGE implantation and one chronic subdural hematoma following implantation of LBS-Neurons, described above, no anatomic or structural changes were noted with any cell therapy on follow-up MRI. There was no evidence of edema, contrast enhancement, mass effect, or change in the appearance of the infarct following treatment with LBS-Neurons. Notably, significant differences were seen in [^{18}F] fluorodeoxyglucose (FDG) uptake on PET scans at 6 and 12 months postoperatively. At baseline, all patients in the phase I study of LBS-Neurons showed marked focal hypometabolism corresponding to the MRI-defined stroke territory. Ipsilateral hypometabolism was variably observed, as was cerebellar diaschisis. At 6 months, >10 % increase in relative FDG uptake in the stroke area was observed in 7 of 11 patients; this increase was

sustained at 12 months in 3 of 11 patients. By 12 months, 5 of 11 patients had at least one postimplantation scan demonstrating a >10 % rise in relative glucose metabolism over baseline. Interestingly, one patient did demonstrate paradoxical decrease in thalamic stroke and surrounding area after implantation. When 6- and 12-month data were pooled, postimplantation increase in metabolic activity relative to baseline in the stroke and surrounding regions significantly correlated with results on the motor subscale of the ESS ($p=0.02$ and $p=0.006$, respectively). No difference in patients receiving two or six million cells was noted. Decreases in the magnitude of contralateral cerebellar diaschisis, which was identified in a majority of cases, also correlated with clinical functional improvement in NIHSS and ESS scores ($p=0.009$ and $p=0.02$, respectively) (Meltzer et al. 2001).

Rather than undergoing PET scans, patients receiving autologous bone marrow therapy were followed using EEG, MR spectroscopy, SPECT, and TMS. At baseline, EEG showed epileptic-like activity in 3 of 5 cases and ipsilateral slow activity in all cases. At 6 months and 1 year postoperatively, epileptic-like activity was detected ipsilaterally to the lesion in all five cases, though no clinical seizures were ever observed. MR spectroscopy showed no significant increase in the N-acetyl-aspartate/creatine ratio when compared to baseline at 6 and 12 months. Temporal changes were noted, however, with a decrease in the immediate postoperative period that reached a nadir at 3 months and then recovered by the 1-year follow-up. Functional mapping using TMS and assessment of regional blood flow using SPECT yielded no significant changes on follow-up imaging.

11.7 Discussion

To date, four small early phase clinical trials of cell therapy for patients with motor deficit from stroke lasting >3 months have been reported. The main objective of these early phase studies was to demonstrate safety and feasibility of cell therapy for stroke patients, with secondary goals of establishing efficacy. Results of these studies suggest that allogeneic transplant of cultured neuronal cells and autologous transplant of bone marrow are safe and feasible, both with respect to the surgical procedure and on long-term follow-up (Kondziolka et al. 2000, 2005; Suárez-Monteagudo et al. 2009). Xenografted porcine fetal LGE cells exhibited less favorable results (Savitz et al. 2005).

Establishment of efficacy, assessed using various scales that measure neurologic and functional deficit resulting from stroke, has proved more elusive. Trials of LBS-Neurons demonstrated some improvement in ESS for patients receiving cells. Interestingly, the group of patients receiving five million cells had both a greater number of ischemic strokes and a greater trend to improvement early on. Authors also observed significant increase in FDG uptake on PET scan. Positive results, however, were tempered by the inability to compare treatment groups due to small sample size and the inconsistency of improvement. Though some patients improved, nearly as many showed no change and some even declined in functional status. Attempts to establish efficacy after bone marrow transplantation ran into similar

problems. Though improvement was noted in some measures of functional deficit (namely, motor), results of other functional tests were not as positive. Additionally, neuropsychological testing suggested possible improvement but provided mixed results that were difficult to interpret.

Given the nature of early phase clinical trials, small sample size was a limitation in all of the studies described; particularly with regard to small, randomized trials, it is difficult to draw conclusions from comparisons of intervention and control groups. Additionally, uncertainties regarding optimal patient characteristics, intervention, and study design abound. Despite some initially promising results, the success of cell therapy for stroke is determined by a myriad of factors, including stroke characteristics (anatomy, type, timing), cell type and mechanism of action, and technical considerations, such as method of delivery, dose, choice of outcome measure, and use of immunosuppression (Bliss et al. 2007; Locatelli et al. 2009; Savitz et al. 2004), none of which have been optimized in clinical applications.

Thus far, all clinical trials of intracerebral cell therapy have been in "chronic" stroke patients or those who continue to have functional deficit for greater than 3 months following onset of stroke. Choice of such patients is likely one of practicality; given the invasive nature of the procedure, safety trials of direct intraparenchymal cell delivery require stable patients with no reasonable expectation of spontaneous natural recovery. Translation of data from preclinical models to chronic stroke patients, however, is problematic. Particularly without strong primate data, extrapolation of results from rat studies is difficult, given the predominant use of allografted human cells, concern for immune rejection, and short rodent lifespan (Kalladka and Muir 2011).

Clinical studies of cell therapy for stroke remain in the nascent phase. As preclinical work evolves, with identification of promising cell types and further elucidation of mechanism of action, so too will translation to the clinical realm. Ongoing trials, such as with SB623 cells, will continue to shed light on the ability of intracranial cell delivery to safely translate into the clinical realm and convey therapeutic benefit. Intracerebral delivery of cells remains a viable and promising option, with great potential for future clinical endeavors.

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Intravenous Cell Therapies for Stroke

12

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12.1 Introduction

The application of cell-based therapies is an emerging technology for cerebrovascular disorders, where there is unfortunately an urgent public health need for new treatments due to the limited endogenous regenerative capability within the brain (Williams and Hare 2011; Chen et al. 2003). For acute ischemic stroke, the predominant cerebrovascular disorder that is the leading cause of adult disability, tissue plasminogen activator is the only approved therapy which promotes recanalization of occluded cerebral arteries; however, only a minority of patients are eligible to receive it (Kleindorfer et al. 2008) because the drug must be administered within 3–4.5 h after symptom onset, according to regulatory guidelines. Once damage from stroke has maximized, little can be done to recover premorbid function. There are no approved effective treatments to reverse or repair brain damage associated with stroke. New therapeutic approaches using cells, rather than drugs, show much promise to promote repair of the injured brain. Among the various types of "cell therapies," there are different kinds of cells that fall into the categories of embryonic, fetal, and adult cell types, all of which are under development as potential new treatments for stroke. A growing body of extensive animal data suggest that cell therapies derived from a range of tissues (whether they are embryonic, fetal, or adult) improve neurological outcome in rodent models of stroke (Mattle and Savitz 2011; Savitz et al. 2011; Honma et al. 2006; Onda et al. 2007).

In this chapter, we discuss the intravenous delivery of cell therapies for stroke. There are currently multiple early phase studies in progress employing different routes of administration (intravenous, intra-arterial, intrathecal, and direct intracerebral transplantation). In stroke, there is an increasing emphasis on the intravenous use of cells for the following reasons: Intravenous (IV) administration is the least invasive and most practical among delivery routes and the most common and safest

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route for drug delivery. After stroke during the post-ischemic inflammatory response, there is upregulation of adhesion molecules, cytokines, and chemokines such as elevated SDF-1, which potently attract inflammatory cells and stem cells to the site of injury (Guzman et al. 2008). The chemotactic signals operating during inflammation and emanating from the brain can be leveraged to direct some types of intravenously injected cells to the damaged areas within the CNS. The intravenous delivery of various cell types has been reported to activate several different signaling mechanisms such as neuroprotective, immunomodulatory, and repair-enhancing processes in the brain. An intravenous administration could therefore lead to widespread cell distribution and consequent secretion of neuroprotective, proangiogenic, and immunomodulatory factors (Guzman et al. 2008). Lastly, there is an emerging literature that cells may exert potent effects on the immune response to stroke within peripheral tissues. Peripheral organs may be key therapeutic targets of systemically injected cells in animal stroke models.

12.2 Mechanism of Action of Stem Cell Therapy in Stroke

Multiple mechanisms of actions have been described after intravenous administration of cell therapies which we review here in more detail. These mechanisms include the following:

Neurogenesis and effects on astrocytes, *oligodendrocytes*, *and axons*: Some types of cell therapies stimulate the brain parenchyma to secrete neurotropic factors such as basic fibroblast growth factor and brain-derived neurotropic factor which activate pathways leading to enhanced survival, proliferation, differentiation, and migration of neural progenitor cells (Zhang and Chopp 2009). Some cell therapies decrease the astrocyte production of neurocan, which is an axon growth inhibiting proteoglycan, and some studies have reported an increase in axonal density around the ischemic lesions in the brain. An increase in the progenitor oligodendrocytes has been seen at the site of ischemic lesion after cell therapy which may enhance myelination. Hence, these mechanisms may play a role in the regeneration and repair process of cell therapy in ischemic stroke (Zhang and Chopp 2009).

Angiogenesis: Both angiogenesis and neurogenesis are closely interrelated and have been observed in the brains of patients with stroke. In a small study, there was a positive correlation between microvessel density and patient survival (Krupinski et al. 1994). There is increased synthesis of angiogenic growth factors such as FGF-2, PDGF, and VEGF and their receptors in the brain after stroke (Font et al. 2010). Injured brains after stroke in animals have shown an association with an increased level of these factors and increased angiogenesis (Zhang and Chopp 2009). Angiogenesis is directly linked to neurogenesis. The later needs new vasculature for prolonged survival. The mechanisms of angiogenesis are similar to neurogenesis and both processes share common factors. Both processes occur in the adult brain as a response to injury but can be stimulated by different types of cellular therapy (Font et al. 2010).

Immunologic mechanism: In rodent models, the adrenergic response post stroke has been associated with the release of immunological cells from the spleen which

contribute to secondary injury and exacerbation of the ischemic lesion. Intravenous umbilical cord blood cells prevent splenic release of immunological cells and decrease secondary injury in the brain (Walker et al. 2010). Different types of cell therapies have been shown to express anti-inflammatory cytokines which may even reduce brain damage due to post-stroke inflammation (Guzman et al. 2008).

12.3 The Perplexing Issue of Cell Trapping and CNS Entry

Despite extensive data attesting to brain remodeling that occurs after intravenous delivery of various types of cell therapies, the extent to which any intravenously administered cell type enters the brain has been a perplexing issue. Our studies and others have found that an intravenous administration of various different types of stem cells leads to their trapping in the lungs (Fischer et al. 2009). Cells lodge in the lungs temporarily and then migrate to other organs such as the spleen (Gao et al. 2001; Schrepfer et al. 2007). Cell size is a clear factor associated with lung trapping (Schrepfer et al. 2007; Harting et al. 2009) as many different types of purified and cultured stem cells have a cell size that is greater than the diameter of the pulmonary capillaries. Internal organs express high levels of SDF-1α, which can also direct cells to selectively home to their sites as well (Kucia et al. 2005). Adhesion molecules on the cell surfaces of capillaries may be another factor promoting lung trapping. Animal studies have shown that inactivation of the counter-ligand for VCAM-1 and CD49d significantly increases the passage of cells through the lung (Fischer et al. 2009). The redistribution of the cells after lung trapping is predominantly seen in the spleen and liver, to such an extent that it cannot be explained on the basis of cardiac output alone. Hence, it is presumed that pulmonary trapping of the cells might alter their ability for tissue homing, such that they migrate in increased numbers to the reticuloendothelial system (Fischer et al. 2009). We have identified that nitric oxide may be an important mediator that facilitates passage of bone marrow cells through the lungs by possibly stimulating vasodilation (Kasam et al. 2012).

Several investigators have begun to unravel the mystery how cells trapped within peripheral organs can still cause such profound effects within the brain. In an animal study of myocardial infarction, pulmonary passage of MSCs upregulated expression of multiple genes, with a large increase in the anti-inflammatory TGF-6 protein (Lee et al. 2009). There is also evidence from both human and animal studies that MSCs do not need to necessarily enter the injured area as they are capable of secreting paracrine factors which are responsible for anti-apoptotic actions leading to recovery (Mezey 2011).

MSCs are thought to exert multiple mechanisms in which they promote recovery. They may interact with immune cells in the reticuloendothelial system. They might induce or inhibit migration of different immune cells into the brain. We have found evidence in our early phase clinical studies that the spleen contracts in patients with acute ischemic stroke. At least in animals, the spleen releases immune cells into the circulation which migrate to the brain. This process might be affected by MSCs within the spleen and reticuloendothelial system. MSCs are thought to “sense” and

change their environment from a pro-inflammatory to anti-inflammatory milieu. They have been shown in animal models of GVHD to induce pro-inflammatory macrophages to become anti-inflammatory by secreting TGF-β and recruiting regulatory T cells (Mezey 2011). Preclinical studies have also shown that mesenchymal cells are effective in reducing lung injury from endotoxin, live bacteria, bleomycin, and hyperoxia (Matthay et al. 2010). Even neural stem cells when administered by IV routes have been shown to downregulate the inflammatory response emanating from the spleen in a model of intracerebral hemorrhage. Thus, multiple types of cell therapies may converge on the peripheral immune response (Lee et al. 2008).

12.4 Cell Types Under Investigation for Intravenous Delivery

Given more than a decade of research on cell therapies in rodent stroke models, a small number of clinical trials testing cell-based therapies in patients with ischemic stroke have been completed, and several more are underway or being planned. The most common cell types that have been brought forward to clinical trials using an intravenous route of delivery (Savitz et al. 2011) thus far are derived from the bone marrow and fall into two major categories. The first is the mononuclear fraction of bone marrow and the second is a more purified, cultured mesenchymal stem cell (MSC) population. Each has their own unique benefits and drawbacks. Mononuclear cells (MNCs) are composed of a mixture of myeloid, lymphoid, and stem cell populations (hematopoietic, mesenchymal, and endothelial); they can be rapidly prepared within hours of a harvest, do not require cell culture, and thus permit autologous administration, avoiding the potential for immunological rejection, a concern with the use of allogeneic cells (Savitz et al. 2011). MSCs, on the other hand, are more homogenous cell types derived from the mononuclear fraction, and their therapeutic applications in neurological disorders are highly supported by a large body of animal literature given their remodeling effects within the brain. MSCs need to be cultured and passaged for scaling to meet the requirements for a clinical trial. The culture conditions and number of passages can alter their biological properties. To date, it has not been possible to culture sufficient numbers of MSCs for an autologous application in the acute or subacute setting of stroke. Consequently, the first trials using autologous MSCs have been tested in patients with chronic stroke. Overall, we will first focus our discussion on early phase clinical studies involving stroke patients receiving intravenous cell therapies in which there have been four published clinical studies (Savitz et al. 2011; Lee et al. 2010; Honmou et al. 2011; Bang et al. 2005; Bhasin et al. 2011).

12.5 Clinical Trials: MSCs

Among the MSC clinical studies, all are from Asia and involve autologous applications. All were pilot trials and hence focused on safety and feasibility of administering intravenous stem cells to patients with ischemic stroke. Overall, there were no signals of safety concerns in any of these trials.

In South Korea, Bang and colleagues published an initial report on 5 patients given their own MSCs compared with 25 patients who were not given MSCs. The rationale for the number of patients in either group was not given. These patients had to have an ischemic stroke within 7 days prior to enrollment, and then they underwent a bone marrow harvest followed by MSC isolation and scale-up in culture. The MSCs were prepared in fetal bovine serum and took on average 30 days to grow to sufficient quantities for autologous infusion. Two doses of autologous MSCs were administered, the first at 4–5 weeks and the second dose at 7–9 weeks. The study patients were followed up to 1 year for safety evaluation. Subsequently, Lee et al. from the same group in 2010 describe the same patients with longer term follow-up and enrollment of more patients, totaling 16 study patients and 36 controls (Lee et al. 2010). There was blinded randomization to the two groups along with blinded outcome assessments. The control patients did not receive any additional interventions aside from standard of care. Twenty-one patients in the control group and 4 patients in the MSC group died but there was no statistically significant difference, although there was a trend toward decreased mortality in the stem cell group. There was no significant difference in the incidence of adverse reactions between the two groups. Of note, there were 5 patients who developed seizures in the control group and 3 in the MSC group. Similarly, there were 3 cases of recurrent vascular events in the controls including 2 with myocardial infarction and 1 with stroke, whereas there were 4 cases of recurrent vascular events in the MSC group out of which 2 were strokes and 2 were myocardial infarctions. Functional outcome on the mRS scale measured in the controls at a median time point of 3.5 years ranged from 2.7 to 4.9, whereas in the MSC group, it was measured at a median time of 3.2 years and ranged from 1.5 to 4.7. The number of patients in this trial was too small to draw any conclusions except that the study intervention appeared safe. Of note, no adverse effects related to fetal bovine serum were observed in any of the patients in the MSC group.

An observational study was then performed in Japan by Honmou and colleagues (2011) in which they enrolled 12 patients who had an mRS of 3 or greater, supratentorial strokes within the prior 6 months, no severe impairment of consciousness (as defined by Japan coma scale of between 0 and 100), and had an age between 20 and 75 years old. Patients who had extensive hemorrhagic transformation, infratentorial strokes, and any other organ dysfunction or severe medical comorbidities were excluded. The patients ranged from 41 to 73 years old with an average age of 59. The NIHSS varied from 2 to 20. Study patients were administered MSCs anywhere from 36 to 133 days after stroke. There was also variability in the dose of MSCs 0.6×10^8 to 1.6×10^8 cells per patient. A notable difference from other trials was that the cultured MSCs were grown in human serum, not fetal bovine serum. Cell passage was limited to three, and infusion occurred over 30 min. The main outcome assessed was safety as measured by neurological worsening, adverse reactions, and evidence of tumor or abnormal growth on MRI, none of which occurred. Clinical outcome assessed by unblinded physicians was measured by serial NIHSS and mRS just prior to cell infusion, immediately after cell infusion, and at several time points after infusion. At 1 year after infusion, the NIHSS ranged from 0 to 5 and the mRS ranged from 1 to 3. Eight out of the twelve patients achieved an mRS of 0–2 at

1 year. Radiological outcome was also measured by serial MRI scans which were interpreted by unblinded radiologists. Based on their assessments, mean lesion volume was reduced by >20 % at 1 week post infusion. This study adds further evidence for the safety of IV administration of MSCs in stroke patients.

Another small study on autologous MSCs was recently published by Bhasin et al. (2011) in India, comprising 12 patients with ischemic stroke within the prior 3–12 months. NIHSS score of enrolled patients was between 4 and 15. Patients were deemed eligible if they were able to comprehend. Patients were excluded if they had bleeding disorders, chronic liver and/or renal failure, progressive neurological worsening, unilateral neglect, neoplasia, contraindications to MRI, and immunosuppression. The selected 12 patients were divided up into 2 groups; half of them served as controls, while the other 6 received IV MSCs derived from their own bone marrow. The patients were followed at 8 and 24 weeks post infusion with laboratory, clinical, and radiological parameters to evaluate for safety. There were no differences clinically in the two groups.

12.6 Clinical Trials: MNCs

In contrast to MSCs, mononuclear cells represent a mixed cell population within the mononuclear fraction of bone marrow. Several randomized controlled clinical trials have reported that MNCs improve ejection fraction in patients with myocardial infarction. Various laboratories have published that MNCs when administered systemically improve neurological outcome in rodent stroke models.

Savitz et al. published a trial on the safety and feasibility of autologous bone marrow-derived MNCs in 10 patients with acute ischemic stroke. Patients were enrolled within 24–72 h of symptom onset, a time window which was felt to be the optimal window for efficacy based on animal studies. Patients underwent a bone marrow harvest (2 ml/kg draw) and then received an intravenous administration of purified autologous MNCs. As this was a safety and feasibility study only, there was no randomization and all patients enrolled received back their own cells. Outcomes were assessed at predetermined time periods at hospital discharge and then at 30, 90, and 180 days. The target maximum dose was ten million cells/kg. Eight out of ten patients received the target dose, but the other two received doses of seven million cells/kg and 8.5 million cells/kg, which represented the highest amount obtained from the harvest. Average age was 55 ± 15 years. There were no severe adverse events associated with the bone marrow harvest or infusion.

Collectively, these studies have begun to provide the first levels of evidence for safety of bone marrow-derived cells in patients with ischemic stroke. The MSC trials involved patients with chronic stroke, while the MNC trial involved patients with acute stroke.

There are a few other ongoing clinical trials of allogeneic MSC trials. Some of them involve chronic stroke and require the patient to have a stroke within the last 6 months, whereas others include patients with acute ischemic strokes. A list of trials can be found on clinicaltrials.gov.

Finally, the Athersys trial is a phase I/II dose escalation study, testing multipotential progenitor adult cells (MultiStem), another adherent stem cell population derived from the bone marrow of healthy volunteers. This study is ongoing and is in the early stages of development for stroke.

12.7 Major Issues That Need Further Study

12.7.1 Cell Type

What types of cell therapies are most suitable or appropriate for IV administration? At the present time, bone marrow and umbilical cord cells are the most conducive for IV. Various cells may exit the bone marrow and home to the brain after stroke. There is therefore an established endogenous mechanism already in place to support IV injections of bone marrow. Some have tested IV neural cells such as neural stem cells in rodent models of stroke (Zhang et al. 2004). Concerns do need to be addressed for the potential of NSCs to become trapped in the lungs and deposit in other peripheral organs.

12.7.2 Selection of Patients

What kind of patients should be included in cell therapy trials involving an intravenous delivery route? All trials thus far have focused on moderate to severe strokes defined either by the NIHSS or mRS. For example, the Japanese MSC study enrolled patients with NIHSS as low as 2, but the mRS was ≥ 3. All the trials restricted the upper age limit of the patients, at 75 in the Japanese and Korean studies, whereas Savitz et al. enrolled up to 80 years of age. As the aging population continues to grow, should we restrict the upper age cut offs? Patients with pulmonary and liver disease will likely need to be excluded at least in early stage safety testing as there is risk of exacerbating pulmonary diseases with entrapment of stem cells in pulmonary circulation.

12.7.3 Timing of Cell Therapy

Another major issue is when to enroll after stroke. The three studies varied greatly from acute stroke transfusion of MNCs by Savitz et al. (2011) to 1-year infusion of MSCs by (Lee 2010; Savitz et al. 2011; Bang et al. 2005), while the Japanese study by Honmou (2011) had a time interval somewhere in between (Honmou et al. 2011). The timing of cell administration should depend on the goal of treatment which spans several different areas from cytoprotection and immunomodulation to neuroregeneration/neurorepair. Some types of cell therapies may engage both sides of the spectrum. Animal studies would seem to suggest that if the former is the principal

goal, then the first few days to weeks is the optimal period. There is an evolving literature that some types of bone marrow preparations such as MNCs or umbilical cord cells when delivered intravenously have an outer limit of efficacy within the first few days (Yang et al. 2011; Iihoshi et al. 2004). Whether more purified cell types such as MSCs have longer windows when administered intravenously is an important issue. Some studies suggest that human MSCs can improve recovery even when given up to 30 days after stroke in rodents. This discussion raises the critical issue that the selection of a time window should be partly based upon animal data.

12.8 Efficacy in Clinical Trials

All clinical trials have been very small exploratory studies. Their main focus was safety and there was no definite study-related severe adverse reaction in any of them. Clinical outcomes were assessed differently in each of the studies, and again given the small number of patients involved and lack of controls, it is premature to offer any comments on efficacy at this time.

Conclusion

Intravenous delivery of cell therapies for stroke is a very practical method which is minimally invasive and supported by extensive animal data. To date, clinical trials have not identified clearly related severe adverse events after the intravenous administration of cell therapies in early phase clinical studies.

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Intra-arterial Cell Therapy in Stroke Patients

13

Gabriel R. de Freitas and Rosália Mendez-Otero

13.1 Introduction

In recent years, several studies have investigated the potential neuroprotective and restorative role of stem cells from different sources in animal models of brain ischemia (Mendez-Otero et al. 2007; Bliss et al. 2010; Hess and Hill 2011). Although the mechanisms of action are still unclear, most of these studies demonstrated that stem cell administration ameliorates the functional loss observed after ischemia, making stem cell transplantation an attractive approach to restore brain function after stroke in humans.

To date, only a few clinical studies evaluated stem cell transplantation in stroke patients. In most of these, stem cells were administered by intravenous (IV) route (Bang et al. 2005; Lee et al. 2010; Honmou et al. 2011; Savitz et al. 2011) or stereotactic surgery (Kondziolka et al. 2000; 2005; Savitz et al. 2005; Suarez-Monteagudo et al. 2009), and stem cells from different sources were used in different times after the stroke which makes comparisons among them difficult.

The aim of this chapter is to review the pilot trials of stem cells administered via intra-arterial (IA) route in patients with ischemic stroke and to discuss the advantages and shortcomings of this approach.

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13.2 Advantages of the Intra-arterial Route

There are scarce data from preclinical and pilot trials in humans to support one route of administration over the others, for example, IA administration over IV route or direct implant (stereotactic surgery) in ischemic stroke. Moreover, the extrapolation of data acquired from the experience with other therapeutic modalities (e.g., thrombolysis) is complex and cannot directly be translated to cell therapies.

Over the past 10 years, the safety and exequibility of IA cell delivery has been investigated in patients with cardiac and peripheral vascular disease, and in most of them no significant adverse events attributed to the cell infusion were reported (Misra et al. 2012). The most important theoretical advantage of the IA route is the larger amount of stem cells in the ischemic and peri-ischemic area after direct injection in the affected artery rather than in the peripheral vein. Studies of thrombolytic therapy for ischemic stroke suggest that IA thrombolysis may be more effective than IV to recanalize large vessels, due to the higher amount of the thrombolytic drug in direct contact with the thrombus when the IA route is used (Mattle et al. 2008). However, when it refers to the delivery and homing of stem cells, the issue is disputable. In animal models, while Kamiya et al. (2008) reported that IA delivery led to a greater brain homing and functional progress in comparison to IV infusion in a model of transient ischemia, Vasconcelos-dos-Santos and collaborators (2012) found that both the IA and IV injections promoted comparable functional improvement with low and similar brain homing in a model of permanent ischemia. Moreover, different groups suggested that the effect of cell therapy in stroke may not be directly related to the presence of cells in the brain, since it was possible to observe functional recovery without the presence of cells in the cerebral parenchyma (Borlongan et al. 2004) or with very few cells present, as shown in studies using different types of cells after IA or IV administration (Bacigaluppi et al. 2009; Brenneman et al. 2010; Gao et al. 2001). To explain these results, it was suggested that the injected cells independent of the route could produce and release trophic factors such as brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and vascular endothelial growth factor (VEGF). These factors could cross the blood–brain barrier and promote the beneficial effects observed in these animals (Borlongan et al. 2004).

In addition, it has been postulated that the trapping of the cells in the lung after IV injection ("the lung barrier") could represent a disadvantage of the IV over IA therapy since the number of cells reaching the brain would be decreased in this case. IA administration bypasses the filter of the peripheral organs including the lungs and directs most of the injected cells into the ischemic tissue. The increase in cell trapping in the lungs after IV injections was actually demonstrated in studies in animal models, which showed a greater uptake in the lungs when this route was compared with IA (Schrepfer et al. 2007). However, it is important to note that different cell types might behave differently in respect to the lungs. For example, a study in normal rats using different cell types found that bone marrow mononuclear cells (BMMCs) passage was 30-fold greater when compared to mesenchymal stem cells (MSCs) and the differences in cell trapping were attributed to the smaller size of the BMMCs when compared to the MSCs and to differences in the adhesion capabilities of both cell types (Fischer et al. 2009). On the other

hand, while pulmonary activity may be seen as a negative factor because cells reach the arterial circulation in smaller proportions, it should be noted that studies in an animal model of myocardial infarction have shown that MSC trapped in the lung secreted tumor necrosis factor-inducible gene 6 protein (TSG6), which decreased myocardial damage and increased myocardial function (Lee et al. 2009). It remains to be investigated whether this passage through the lungs as well as other organs such as the spleen could be instructive to the injected cells or not and whether this passage could result in changes in the functional capabilities of the injected cells.

13.3 Limitations

The major disadvantage of the IA administration is the fact that it is an invasive procedure with a small, but non-negligible, risk of complications. A retrospective study reviewing cerebral angiographies in nearly 20,000 patients indicated that the risk of complications due to the procedure is approximately 2 % and that the risk of a new stroke is approximately 0.14 % (Kaufmann et al. 2007). In addition to the intrinsic risks of a cerebral angiography, it is possible that the injection of cells may increase the risk of ischemia due to the occlusion of microvessels or capillaries. A preclinical study using MSCs found that despite the benefits of IA delivery of stem cells to the ischemic brain, there was a clear risk of vascular occlusion and also an increase in mortality compared to non-transplanted animals (Walczak et al. 2008). In fact, in one pilot trial of IA administration of BMMCs in acute stroke that included seven patients, the only complication was an embolic stroke in one patient during the angiographic procedure but before the administration of the cells (de Freitas et al. 2006). The patient did not receive the BMMCs and presented neurological deterioration. The fact that no other patient in the pilot clinical trials presented new ischemic lesions on diffusion-weighted (DWI) magnetic resonance imaging (MRI) after injection of the cells argues that the complication is linked to the procedure, not to the cells per se. Another shortcoming is the requirement of the patency of the intracranial and extracranial circulation (e.g., significant stenosis or occlusion was an exclusion criterion of all the pilot studies). In our view, the occlusion of an intracranial artery but with reasonable collateral supply should not be considered a definitive contraindication to IA administration, since in these cases even with more proximal injections, cells would still be able to reach the damaged area through collateral circulation. Finally, another disadvantage of the IA route is the contact of the cells with the contrast used in the arteriography since results from our group have shown that the viability of the cells decreases in the presence of the contrast (unpublished results).

13.4 Pilot Clinical Trials

At the time of writing this chapter, only five trials of stem cell administration for ischemic stroke using IA delivery were reported in the literature (de Freitas et al.

2006; Battistella et al. 2011; Friedrich et al. 2012; Moniche et al. 2012; Banerjee et al. 2012), two of them in abstract form (de Freitas et al. 2006; Banerjee et al. 2012) (Table 13.1).

In 2006, our group reported in the International Stroke Conference the results of a small pilot trial designed to evaluate the safety and feasibility of IA transplantation of autologous BMMCs in patients with acute middle cerebral artery (MCA) ischemia (de Freitas et al. 2006). Inclusion criteria were age between 18 and 80, a score between 4 and 20 in the National Institutes of Health Stroke Scale (NIHSS), and administration of the cells within 7 days of stroke onset. Seven patients (two women) with a mean age of 49 years (range from 38 to 58) and mean NIHSS of 9.5 were included in the trial. Bone marrow cells were aspirated (50 ml) from the posterior iliac crest under local anesthesia, and BMMCs were isolated by density gradient on Ficoll-Paque Plus, resuspended in saline with 5 % human serum albumin and immediately injected (30×10^6 cells in 10 ml) in the MCA under continuous transcranial Doppler and electroencephalographic monitoring. The infusion was done at the rate of approximately 1 ml/min. Patients underwent anticoagulation with intravenous heparin to obtain an activated clotting time of two to three times baseline. Differently from cardiac studies that performed intracoronary administration of the cells, a balloon catheter was not used to occlude the target MCA and increase cell delivery because of the risk of arterial dissection. One patient exhibited neurological deterioration (recurrent embolization) during arteriography, did not receive the cells, and was excluded from further analysis. The six patients treated exhibited an uneventful subsequent clinical course, with no new lesions appearing in sequential DWI MRI exams (D7, D60, and D120) and no sign of embolization or epileptic activity. Mean NIHSS scores varied from 9.5 (entry) to 4.5 (D60) and 3 (D120). The corresponding modified Rankin Scale (mRS) and Barthel Index (BI) scores were 3.5, 2, and 2 and 52.5, 92.5, and 52.5, respectively.

To evaluate the feasibility of monitoring the cells implanted into the brain, in one of the patients, approximately 1 % of the 3.0×10^7 BMMCs delivered into the left MCA were labeled with 150 MBq (4 mCi) Technetium-99 m (Tc-99 m) by incubation with hexamethylpropylene amine oxime (HMPAO) (Correa et al. 2007). Tomographic views of the brain, obtained 8 h after BMMC-labeled cell delivery, revealed intense accumulation of the cells in the ipsilateral hemisphere. A whole-body scan was done and showed left brain, liver, and spleen uptake. The results suggested that brain SPECT imaging with labeled cells could be a viable noninvasive method for studying the fate of transplanted cells in vivo.

To address the feasibility of the IA administration of BMMCs after the first few days of ischemic stroke (nonacute stroke phase), Battistella et al. (2011) design a pilot trial with similar characteristics and inclusion criteria of the previous study but including patients up to 90 days after the stroke onset (NCT00473057). Another difference from the previous trial was that in all patients approximately 10 % of the BMMCs were labeled with Tc-99 m and scintigraphies were carried out 2 and 24 h after the procedure to analyze cellular homing and biodistribution. Six male patients, with a mean age of 69 years (range from 24 to 65 years), received between 1.25×10^8 and 5×10^8 BMMCs via IA administration from 59 to 82 (mean 69) days after MCA

Table 13.1 Reported clinical trials of stem cells administered by intra-arterial route in patients with ischemic stroke

First author, year	Number of patients (men, women)	Mean age (range)	Mean NIHSS	Artery involved	Cell type	Number of cells	Time of infusion, mean (range), days	Control group	Complications	Monitoring of cell fate
de Freitas (2006)	6 (4 M, 2 W)	49 (38–58)	9.5	MCA	BMMC	3×10^7	NR (3–7)	No	One ischemic stroke during angiography	Only one patient, Tc-99m
Battistella (2011)	6 (6 M, 0 W)	51 (24–65)	9	MCA	BMMC	Range 1.25×10^8–5×10^8	69 (59–82)	No	Seizures in two patients	All patients, Tc-99m
Friedrich (2012)	20 (14 M, 6 W)	63 (30–78)	17	MCA	BMMC	Range 5.1×10^7–60×10^7	6 (3–10)	No	–	No
Moniche (2012)	10 (5 M, 5 W)	66.9 (13.9[a])	15.6	MCA	BMMC	Mean 1.59×10^8	6.4 (±1.3[a])	Yes	Seizures in two patients	No
Banerjee (2012)[b]	5 (NR)	NR	NR	MCA	CD34+	NR	Within 7 days[c]	No	–	No

BMMC bone marrow mononuclear cells, *M* men, *MCA* middle cerebral artery, *NR* not reported, *Tc-99 m* Technetium 99 m, *W* women
[a]Standard deviation
[b]Ongoing clinical trial
[c]Planned

infarcts, with a mean NIHSS of 9 (range from 4 to 13) at the time of cell infusion. No signs of worsening in the neurological condition were observed immediately after the procedure or during the follow-up period. At the 180-day follow-up evaluation, all patients had improved their scores in comparison with the values before transplantation. For example, the NIHSS scores improved (range from −1 to −8 points) during follow-up in all patients. Two patients suffered generalized seizures after the end of follow-up (around 200 days after the BMMC infusion); one was successfully treated with phenytoin, and the other was treated with a combination of oxcarbazepine and lamotrigine. Whole-body scans obtained 2 h after transplantation of labeled BMMCs showed uptake in the brains of all patients, which ranged from 0.6 to 5.1 % when compared to the activity in the whole body. Quantification of cell uptake in SPECT images indicated preferential uptake on the side of the lesion in all patients. Nevertheless, these differences were widely variable, ranging from 58 to 98 % of total brain uptake. The remaining cell uptake was distributed mainly to the liver, lungs, spleen, and kidneys in all patients. Due to the short half-life of Technetium, uptake could only be visualized in the brains of two patients 24 h after injection, while in all patients uptake was seen in the liver, lungs, spleen, kidneys, and bladder (Barbosa da Fonseca et al. 2009; 2010).

A clinical trial with the largest number of patients was recently published. Friedrich et al. (2012) administered the BMMCs IA in 20 patients with more severe MCA infarctions and at an earlier time point than the previous trials (mean time from stroke onset to treatment 6 ± 1.8 days). The mean age was 63 years (range from 30 to 78), 14 were males, and the right hemisphere was the affected side in 12 patients. The mean baseline NIHSS score was 17 ± 5.6 (median 15.5, range from 9 to 28). The mean cell count in the infused solution was 22.08×10^7 cells (range from 5.1 to 10^7–60×10^7). None of the patients met the primary end point focused on safety: clinically significant procedural complications (defined as a decline of ≥4 points in the NIHSS score or death), symptomatic intracranial hemorrhage, new ischemic lesions on computed tomography (CT) and/or DWI MRI at day 7 post procedure, clinical seizures and/or epileptic discharges on serial electroencephalogram (EEG) at any time during the hospitalization, and development of intracranial neoplasia in the long-term follow-up. Eight patients (40 %) showed a good clinical outcome, defined previously as a mRS ≤ 2 at 90 days.

Also recently, the results of another trial were published (Moniche et al. 2012). This trial enrolled 10 patients with severe (NIHSS ≥8) MCA infarction to receive BMMCs transplantation (NCT00761982). The first 10 consecutive patients included were considered the active group, and 10 patients were included in the control group. Although no bone marrow aspiration or sham injection was performed in the control group, neurologists who evaluated the patients at 1, 3, and 6 months were unaware of the treatment allocation. Mean NIHSS was comparable between the two groups (15.6 in BMMC group versus 15.0 in the control group, $p = 0.82$), and transplantation was done at 6.4 (±1.3) days after stroke onset. There were no serious adverse events during the BMMC transplantation procedure. During follow-up, two BMMC-treated patients had an isolated partial seizure at 3 months. There were no

seizures in the control group. Neurological disability at 6 months was not significantly different in the two groups, but there was a trend toward a better outcome when higher numbers of CD34+ cells were injected.

Banerjee et al. (2012) reported in the European Stroke Conference the partial results of their study (NCT00535197). The aim of the investigators is to enroll 10 patients with complete MCA infarctions, isolate and harvest CD34+ by immunoselection, and inject them into the MCA within 7 days of the event. Up to the presentation, five patients had been enrolled with no serious treatment-related adverse events, and all presented improvement in their clinical scores and reduction in lesion volume up to 6 months follow-up.

13.5 Comparison Between Intravenous and Intra-arterial Administration in Humans

The small number of patients and diverse characteristics (e.g., inclusion criteria, type and number of cells injected, time of administration) prevent direct comparison of IA and IV stem cell studies. Recently, Rosado-de-Castro et al. (2012) compared safety end points and biodistribution of cells in patients with subacute ischemic stroke treated in the same hospital, using the same cell type and similar inclusion criteria but different routes. After bone marrow harvesting, approximately 2×10^7 BMMNCs were labeled with 99mTc and delivered via IA or IV together with the unlabeled cells. Scintigraphies were carried out at 2 h and 24 h after cell transplantation. Seven patients were included in the IA group (six of these were already described above in the study of Battistella et al. 2011) and five in the IV group, between 19 and 89 days after stroke. Cell homing in the brain compared to the whole body was low and similar between both routes at 2 h and 24 h. However, when the homing in the ischemic hemisphere was compared with the uptake in the contralateral hemisphere, there was a slightly greater relative uptake of 99mTc-BMMNCs at 2 h in the IA group (68.1 ± 14.6 in the IA group versus 53.4 ± 3.8 in the IV group, $p = 0.023$). The quantification of whole-body images indicated that the IA route led to greater uptake in the liver and spleen and lower uptake in the lungs at 2 h when compared to the IV route. All patients had neurological improvement, with a decrease in the NIHSS ranging from 1 to 10 points during the follow-up period. Two patients of the IA group and 5 patients of the IV group had seizures that were controlled with antiepileptic medication.

Conclusions

IA delivery of stem cells is a promising therapy for patients with cerebral infarcts. Pilot studies in stroke patients suggest that it is safe. Whether homing of stem cells in the affected brain region is larger with IA than with IV therapy is debatable. Limited data from a human study suggest a slightly greater homing of cells in the hemisphere ipsilateral to the ischemia when the cells are delivered IA. However, whether this result translates into greater clinical benefits is unclear

since there are data indicating that functional recovery may occur even in the absence of stem cells in the brain. Moreover, the passage of the cells in peripheral organs may confer them protective properties. Larger, randomized phase III studies are necessary to establish the efficacy of this approach and the best delivery route.

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Intraventricular Route of Cell Transplantation for Stroke-Related Diseases: Rationale, Risk, and Early Clinical Experience

14

Miroslaw Janowski

14.1 Introduction

The central part of the brain is occupied by a fluid compartment. During the early phase of development, a neural plate folds in on itself to form a hollow neural tube, with neuropores on both sides, and is initially filled in with amniotic fluid. The capillary plexus develops around the neural tube, and as neuropores close, the plexus invaginates with the pia mater and ependyma deep into the tube within the thin areas of the neural tube roof (Dziegielewska et al. 2001). The thus-formed choroid plexus begins production of cerebrospinal fluid (CSF), pushing out the walls of the neural tube and forming cerebral ventricles in sites of invagination. Once ventricular system is already shaped, the foramina of Magendie and Luschka, which allow for CSF outflow from the ventricles, inaugurate lifelong CSF circulation. The mature ventricular system consists of four ventricles: two lateral ventricles and the third and fourth ventricles walled off by a thin layer of ependyma and filled in by CSF.

CSF is actively produced by the choroid plexus within the cerebral ventricles (mostly the lateral ventricles) and flows through the entire ventricular system until it runs out via the foramina of Magendie and Luschka into the subarachnoid space surrounding the central nervous system (CNS). Then, CSF is absorbed by arachnoid granulations and lymphatic vessels related to the cranial nerves (Kiwic et al. 1998). Thus, CSF does not return to the ventricular system from the subarachnoid space. Inside the ventricular system, CSF is walled off from the brain parenchyma by a thin layer of ependyma, easily permeable for small molecules, proteins, and even

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cells (Walczak et al. 2007). While in the subarachnoid space, CSF is walled off from the brain parenchyma by the pia mater, a fibrous membrane that forms an impermeable barrier (Weller 2005). Thus, the distinction between the ventricular system and the subarachnoid space is very important from the point of therapeutic targeting of the brain. While substances/cells injected to cerebral ventricles may relatively easily penetrate the brain parenchyma, it is not much expected after their delivery to subarachnoid space.

While the permeable character of the ependyma facilitates the delivery of therapeutics, including cells, to the brain parenchyma, there is an even greater reason to pay specific attention to the lateral ventricles. These ventricles are surrounded by neurogenic, lifelong proliferating zones that generate new neurons for the brain, namely, the subventricular zone (SVZ) and the dentate gyrus (DG) (Alvarez-Buylla et al. 2008; Ihrie and Alvarez-Buylla 2011). The SVZ was initially described in rodents (Lois and Alvarez-Buylla 1993) and then confirmed in larger animals, such as pigs (Guidi et al. 2011), sheep (Brus et al. 2013), and nonhuman primates (Tonchev 2011), and in humans (van den Berge et al. 2011). Recently, DG neurogenesis across species and orders was reviewed extensively (Amrein et al. 2011; Barker et al. 2011). Thus, the close proximity of neurogenic niches to the lateral ventricles is a key to which intracerebroventricular route may play a prominent role in the therapies of neurological diseases. This is because the lateral ventricles are easily selectively targeted, or the entire neurogenic zone can be targeted, neither of which is possible with the intraparenchymal and intravascular routes. In fact, the CSF is already used for natural, endogenous regulation of neuronal differentiation by choroid plexus-derived molecules during CNS development and neuronal repair following injury (Redzic et al. 2005). A stroke-induced increase in endogenous neurogenesis has been reported by many authors (Jablonska and Lukomska 2011; Kernie and Parent 2010; Xiong et al. 2010).

14.2 Rationale

14.2.1 Hypoxic-Ischemic Neonatal Injury

During the second part of pregnancy and after birth, the subventricular zone (SVZ), a highly proliferative zone in the forebrain, has a uniquely important role in providing neurons for the developing brain (Zecevic et al. 2005). However, neurogenesis originating from the cells of the SVZ is significantly reduced after hypoxic-ischemic injury in the neonatal rat brain (Spadafora et al. 2010). It was observed through noninvasive in vivo imaging that endogenous neuronal precursors, which in healthy brains migrate along the rostral migratory stream (RMS), travel toward the lesioned area in hypoxic-ischemic conditions (Yang et al. 2008). Thus, it would be highly desirable to stimulate neurogenesis or provide exogenous cells to supplement the missing cells in the developing brain.

Excellent incorporation and site-specific differentiation of both primary and in vitro-derived neural precursors was observed across species in animal (rodent)

models after transplantation to the embryonic lateral ventricles, generating widespread CNS chimerism with phenotypes indistinguishable from neighboring host cells (Brustle et al. 1997, 1998; Campbell et al. 1995; Peng et al. 2002; Wernig et al. 2004). Then, it was shown that transplantation of neural stem/progenitor cells into the neonatal lateral ventricles also resulted in excellent incorporation of cells into the cytoarchitecture of the recipient brain (Tamaki et al. 2002). Another study showed that human fetal-derived neural stem cells transplanted into the cerebral ventricles of the neonatal rat after hypoxic-ischemic injury reveal excellent survival, migration toward the lesioned area, and differentiation into neurons and glial cells in a regionally specific fashion (Qu et al. 2005). Similar observations were made after the transplantation of human embryonic germ cell-derived neural stem cells, which replaced the neurons and oligodendrocytes in the forebrain of neonatal mice subjected to an excitotoxic ischemia-like brain injury (Mueller et al. 2005). Intraventricular co-injection of neural progenitors with chondroitinase ABC enhanced the therapeutic effect, but, surprisingly, did not increase the number of cells migrating to the lesion (Sato et al. 2008). Co-infusion of neural progenitors with brain-derived neurotrophic factor (BDNF) to the lateral ventricles increased the survival of transplanted cells and positively affected behavioral outcome (Wang et al. 2008). Not only did the neural progenitors reveal positive effects, but also rat mesenchymal stem cells (MSCs) allotransplanted to lateral ventricles resulted in wide migration over the parenchyma and in positive behavioral effects (Liu et al. 2008). A comparison of the methods of transplantation of neural stem cells (NSCs) to the cortex, hippocampus, and lateral ventricles in rat hypoxic-ischemic brain injury revealed that the intraventricular route is the most effective therapeutically (Wang et al. 2007).

Intraventricular administration of insulin growth factor-1 (IGF-1) (Lin et al. 2005), as well as BDNF and nerve growth factor (NGF) (Im et al. 2010), in a rodent model of hypoxic-ischemic injury in neonatal animals (rodents) increased both endogenous neurogenesis and improved behavioral recovery. Thus, overexpression of those factors in transplanted cells might be useful to enhance the therapeutic effect.

14.2.2 Periventricular Leukomalacia (PVL)

In contrast to full-term infants, white matter damage (periventricular leukomalacia) is the predominant form of ischemic injury in premature infants and the most common antecedent of cerebral palsy (Titomanlio et al. 2011). It has been shown that NSCs enter the brain after intracerebroventricular administration, differentiate into oligodendroglia, and improve the outcomes in cases of cerebral white matter damage. It seems that intraventricular transplantation of NSCs is of great potential value for the treatment of PVL in premature infants (He et al. 2008). Oligodendrocyte precursors revealed neuroprotective potential in a subchronic model of PVL (Webber et al. 2009). The intraventricular infusion of the sonic hedgehog (SHH) protein resulted in an increase of proliferating oligodendroglial progenitors (Loulier et al. 2006).

14.2.3 Ischemic Stroke

Neural stem/progenitor cells transplanted to the lateral ventricles are able to distribute widely within the brain parenchyma, differentiate into mature brain cells, and facilitate behavioral recovery (Kim et al. 2004; Ma et al. 2007; Modo et al. 2002). There are also reports of transplantation of nonneural cells into the lateral ventricles in an animal model of stroke, with positive behavioral outcomes. Newborn-derived microglia were able to infiltrate the stroke area (Kitamura et al. 2004, 2005), while no information about the fate of transplanted amniotic epithelial fluid cells was provided (Rehni et al. 2007). It was shown that the therapeutic effect of NSCs could be further enhanced by overexpression of hypoxia-inducible factor-1α (HIF-1 α) (Wu et al. 2010).

There are several substances which, after infusion into the cerebral ventricles, are able to increase neurogenesis, such as activin (Abdipranoto-Cowley et al. 2009), brain-derived neurotrophic factor (BDNF) (Zigova et al. 1998), and neuropeptide Y (NPY) (Decressac et al. 2011). In addition to neurogenesis, behavioral effects after the administration of epidermal growth factor (EGF) and erythropoietin (Kolb et al. 2007) or Ephrin-1 (Jing et al. 2012) were also evaluated in animal models of brain ischemia. Both studies revealed an increase in neurogenesis and positive functional effects. Thus, overexpression of the above factors in transplanted cells may further enhance their therapeutic potential. However, a direct link between neurogenesis and behavioral recovery needs to be confirmed by new technology (Lagace 2012).

14.2.4 Hemorrhagic Stroke

Hemorrhagic stroke is less frequent, but is characterized by a much more severe course and, as such, is of great interest as a target for cell therapy. Embryonic stem cell-derived neurons and astrocytes were observed around the hematoma cavity following intraventricular transplantation (Nonaka et al. 2004). Human amniotic epithelial cells transplanted intracerebroventricularly remained inside the ventricles, but were able to reduce brain edema and improve motor deficits (Dong et al. 2010). Overexpression of hepatocyte growth factor (HGF) enhanced the therapeutic effects of human umbilical cord blood-derived (HUCB)-MSCs via an increase in remyelination and axonal regeneration (Liu et al. 2010). Since, in some patients, cerebral hematomas penetrate the ventricular system and require ventricular drainage placement, those patients could be excellent candidates for the intraventricular administration of cell therapy.

14.3 Risk Associated with Intraventricular Route

While the effects of intracerebroventricular cell transplantation are generally promising, this approach is not devoid of risk. It has been shown that the intracerebroventricular administration of platelet-derived growth factor (PDGF) may produce

glioma-like cell masses that originate from PDGFRα-positive cells in the ependyma (Chojnacki et al. 2011), whereas continuous injection of EGF produces intensive proliferation of periventricular cells (Gampe et al. 2011; Gonzalez-Perez et al. 2009). Cells transplanted to the lateral ventricles in the vicinity of a highly inflammatory milieu penetrate the brain parenchyma and grow into tumorlike masses (Grigoriadis et al. 2011). It is also important to know the secretory profile of the transplanted cells because some substances, such as oncostatin M, rather than having a therapeutic effect may actually cause intellectual deterioration by inhibition of constitutive neurogenesis (Beatus et al. 2011). While most studies revealed positive effects of cell therapy, some studies showed no therapeutic effects (Smith et al. 2012); thus, there is also a risk of giving of unjustified hope for patients.

14.4 Early Clinical Experience

Despite the magnitude of studies that have presented positive results for intraventricular cell therapy for ischemic and hemorrhagic brain injuries in animal (rodent) models, there are only single clinical case reports on the utility of this route for neurotransplantation in humans. One reason might be the lack of experiments in large animals, which, because they provide translational information, are more convincing for clinicians. Below, we present two cases of cell transplantations performed in China and Poland, as well as one relevant case report of the application of growth factor. All of them concern young pediatric patients, and there are no cases in which the intracerebroventricular route of cell therapy for adult stroke patients has been performed.

14.4.1 Chinese Case

Severe hypoxic-ischemic injury in newborns results in lifelong consequences including motor disabilities and cognitive impairment, with no efficient treatment available beyond intensive, costly rehabilitation. Although this complication is infrequent, when the number of deliveries worldwide is considered, there are many newborns awaiting treatment. This was a case of a 75-day-old pediatric patient, with a history of severe hypoxic-ischemic injury at birth, with a significant delay in cognitive and motor functions and accompanying myotonia, who underwent cell therapy (Luan et al. 2005). An MRI scan revealed multifocal cerebromalacia and encephalopathy.

Cells for transplantation were derived from the forebrain of an 11-week-old fetus and multiplied in culture over a 15-day period. After intraventricular injection, the patient was followed over 1 month. Clinical examination revealed substantive improvement of all symptoms, with motor functions recovered to the level of normal infants. A PET scan revealed a significant increase in the metabolism in the occipital and temporal lobes, which was ascribed to the graft activity. No direct imaging of transplanted cells was performed. The Chinese researchers showed that

allografted fetal-derived neural precursors are able to reverse the clinical sequelae of severe hypoxic-ischemic neonatal brain injury. The mechanism of action of transplanted cells remains unknown, but, based on preclinical data, the direct incorporation of transplanted cells into the cytoarchitecture of the host brain should be considered.

14.4.2 Polish Case

Severe, global ischemia in a baby several months old was of iatrogenic origin (Jozwiak et al. 2010). Following resuscitation, the patient regained respiratory action, but was left severely impaired. After 3 months, a persistent vegetative state was diagnosed. An intensive rehabilitation program performed 3 months prior to transplantation did not result in any signs of improvement. Before transplantation, the patient was still severely impaired, unresponsive, and spastic, with generalized epileptic seizures. MR imaging revealed global brain atrophy, without any focal lesion.

Cells for transplantation were derived from the patient's own cord blood, which was stored in a private Polish Stem Cell Bank (PBKM SA). Cells were directed in vitro into a neural phenotype using a 10-day-long differentiation protocol (Habich et al. 2006). The intraventricular route for cell delivery was carefully selected. Since global brain changes were observed on MRI, local intraparenchymal delivery was not thought to be an optimal solution. In addition, it had been several months since the ischemic events occurred, and it was thought that the blood–brain barrier had been resealed and cells might encounter obstacles in reaching the CNS. Thus, an intravascular route was also disqualified. An intraventricular route seemed to have several advantages: the relative safety of the procedure (routine in pediatric neurosurgery), the access of cells to wide areas of the brain, and the close proximity of cells to neurogenic zones, which potentially could be influenced/stimulated by factors released from cells. However, to expect any therapeutic activity from transplanted cells, the cells should remain within the ventricular system, and, if they leave the ventricular system, they could be separated from the brain by the fibrous, impermeable pia mater. Since the CSF circulates from inside the ventricles to the outside, it is of great importance to know whether cells remain in the lateral ventricles and therapeutic effects might then be expected or whether these cells leave the system so that future studies could be adjusted to avoid such undesirable cell outflow. The total number of cells present in a frozen cord blood unit was divided into three equal portions, and these portions were scheduled to be transplanted monthly. To follow the cell distribution, a portion of the cells (20 %) was labeled with iron oxide for the first cell transplantation, which enabled in vivo cell visualization by MR imaging.

Surgery was performed in the supine position using general anesthesia. Under magnetic neuronavigation, a catheter was placed in the frontal horn of the right lateral ventricle, and then 1.2×10^7 cells, suspended in 0.5 ml, were injected. The day after surgery, MR imaging was obtained and compared to the preoperative MR scan. In the occipital horn of the right lateral ventricle, there were visible hypointensities that corresponded to the signal from the iron oxide-labeled cells. Next, MR

scans performed 1 week and 1 month posttransplantation showed iron oxide signal in the same place; however, its gradual decrease was observed. Thus, for the subsequent transplantations, the surgical procedure was not changed, nor were cells labeled with iron oxide. Follow-up MR scans revealed a further decrease in MR signal, until the complete disappearance of signal 33 months later. The first clinical assessment performed by neurologist at the 6-month follow-up time point showed mild improvement. The last clinical examination performed by neurosurgeon at the 33 months follow-up time point did not show further recovery. However, despite the very poor prognosis, the child was alive over the entire period of observation.

Based on this study, transplantation of autologous cord blood-derived cells, labeled with iron oxide, is safe, provides information on cell distribution after injection, and may result in mild clinical improvement. However, a very long time window of several months from the ischemic event to cell transplantation may account for the limited clinical outcome.

14.4.3 Italian Case

Two patients, 8 and 13 months of age, experienced cardiorespiratory arrest followed by a comatose state and were admitted to pediatric intensive care unit (PICU) (Chiaretti et al. 2008). NGF was administered intracerebroventricularly to them at PICU 4 months later, over a 10-day period. An extensive evaluation of patient status was performed pre- and post-NGF infusion, including a neurological examination, EEG, MR, and SPECT. It was shown that the NGF infusion significantly improved the outcome of both patients. The patients began to be expressive, were able to be discharged from the PICU, and subsequently received physiotherapeutic support at home. MR imaging revealed a reduction in encephalomalacia and cystic cavitations. EEG showed a decrease in slow-wave activity. A remarkable increase in radiotracer uptake suggested an improvement in brain metabolism. Additional investigation revealed an increase in doublecortin (Dcx) presence in the CSF of both patients, which suggests stimulation of endogenous neurogenesis by the infused NGF.

The quite impressive recovery of these patients after the intraventricular administration of NGF resembles the preclinical data (Luk et al. 2004; Yang et al. 2011). Taking into consideration the increase of Dcx in the CSF, the enhancement of endogenous neurogenesis is most likely responsible for the therapeutic effects of NGF. Transplantation of NGF-overexpressing cells might be an option to extend brain exposure to this growth factor.

Conclusions

There is a very promising data from preclinical rodent studies on the utility of the intraventricular route for the cell therapy of ischemia and hemorrhagic stroke. Neural progenitors are the most frequently used cell type. Transplanted neural progenitors were easily incorporated into the brain parenchyma. The application of neurotrophic and growth factors or cells expressing them was also confirmed to be a viable therapeutic strategy.

Despite the magnitude of studies that report positive results for intracerebroventricular cell therapy for ischemic and hemorrhagic brain injuries in animal (rodent) models, there are only single clinical case reports on the utility of this route for neurotransplantation in humans. All of them revealed at least mild clinical improvement, with no side effects.

However, there is a paucity of translational studies on large animals, which could contribute to the optimization of transplantation conditions and to a better understanding of the mechanisms that mediate cell-dependent effects.

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