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Review

Tracking stem cell migration and survival in brain injury: Current approaches and future prospects

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Summary. In recent years, stem cell-mediated therapies have gained considerable ground as potential treatments for a wide variety of brain pathologies including traumatic brain injury, stroke and neurodegenerative diseases. Despite extensive preclinical studies, many of these therapies have not been fully translated into viable clinical approaches. This is partly due to our inability to reliably track and monitor transplanted stem cells longitudinally over long periods of time in vivo. In this review, we discuss the predominant histological cell tracing methodologies, such as immunohistochemistry, and fluorescent cellular dyes and proteins, and compare them to emerging cellular imaging technologies. We show that advances in magnetic resonance imaging (MRI) have resulted in opportunities to use this technology to further our understanding of stem cell characteristics and behaviors in vivo. While MRI may not completely replace conventional cell tracking methods in pre-clinical, mechanistic work, it is clear that it has the potential to function as a powerful diagnostic tool for tracking stem cell migration and survival as well as for evaluating the efficacy of stem cell-mediated therapies.

Key words: Stem cells, MRI, Trauma, Stroke

Introduction

Annually, millions of patients are afflicted by a wide variety of injuries and disease that result in neuronal damage and death. Unfortunately, once neuronal death has occurred following traumatic brain injury (TBI), stroke or neurodegenerative disease, there are few available effective treatments. This gap has led to the widespread investigation of stem cell-mediated therapies. However, translation from the lab to the clinic has not been fully realized due, in part, to our insufficient ability to track stem cell migration and survival *in vivo*. This is particularly problematic in a clinical setting where the use of conventional histological techniques is precluded in recovering patients. This review will discuss a variety of conventional histology-based cell tracking techniques and compare them to emerging imaging modalities to track both exogenously administered stem cells as well as endogenous stem cells and neuronal precursors that are induced to proliferate in the adult brain after injury.

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Histochemical techniques

Thymidine analogues

During the DNA synthesis phase of the cell cycle, thymidine analogues are incorporated into newly replicated DNA. It is for this reason that halogenated thymidine analogues such as bromodeoxyuridine (BrdU) can be used effectively to label proliferative cell populations. The ease with which thymidine analogues are incorporated into proliferating cells is an attractive quality and has led to their ubiquitous use as a label for proliferating cells. BrdU, accompanied by histological analysis, has been successfully used track the location and determine the fate of bone marrow derived rat mesenchymal stem cells (MSC) intracranially transplanted in a rodent model of TBI (Mahmood et al., 2002). BrdU has also been used for labeling migratory neural progenitor cells (NPCs) derived from the human forebrain. Here it was shown that BrdU applied to proliferative NPCs in culture could be used to trace their migration behavior and identify their destination up to six weeks after transplantation. BrdU-labeled NPCs

transplanted perilesionally immediately subsequent to brain insult migrated toward the damaged cortical areas (Wennersten et al., 2004).

Despite the valuable information that has been derived from the use of BrdU to track and study stem cells, this method has a number of limitations. In the central nervous system, thymidine analogs can be transferred from pre-labeled donor cells to neighboring host cells (Burns et al., 2006). This represents a major pitfall in the reliability of using labels like BrdU because it can lead to the incorrect identification of host cells as transplanted donor cells. Furthermore, it may reduce the concentration and therefore detectability of the label as it is transferred to surrounding cells. Additionally, stem cells do not asymmetrically segregate their chromosomes (Kiel et al., 2007). Thus, pre-labeled cells will not exclusively retain the label that has been incorporated into their DNA. Instead, the label will be diluted as it is transferred to the daughter cells of the originally labeled progenitor cell. There is also evidence that thymidine analogs slow down cell proliferation regardless of cell type (Michishita et al., 1999). This is a problem when using BrdU and other analogs to measure proliferative rate, or in long-term tracking of stem cells.

Anti-human Immunohistochemistry

In the context of a preclinical animal model, it is possible to exploit species-specific markers. One such strategy can be applied when using donor cells of human origin in a rodent model. In this scenario, it is possible to confidently identify human donor cells engrafted within the rodent host by using antibodies specific to uniquely human antigens. Recently, this technique was successfully employed to confirm the engraftment of human MSC in a rat traumatic brain injury model. To do so, an antibody specific against human mitochondria (E5204, Spring Bioscience, CA) was used to distinguish transplanted hMSC from surrounding host cells (Li et al., 2011). The use of anti-human mitochondrial antibodies has also been successfully employed in differentiating transplanted hMSC from host cells in a traumatic spinal cord injury model (Sheth et al., 2008). Other successful attempts at species-specific immunolabeling include anti-human nuclei (Ravindran and Rao., 2006), anti-human neuronal specific enolase (Zhang et al., 2005).

Y-Chromosome marker

In 2005, Crain et al. used fluorescence *in situ* hybridization (FISH) to successfully visualize the Y-chromosome marker in paraffin brain sections of human female patients who had received either bone marrow or stem cell transplants from male relatives. Co-labeling for neuronal and astrocytic cell markers was successfully observed in sections of the cortex, hippocampus, striatum and cerebellum (Crain et al., 2005). This method has also been successfully employed in animal models of brain injury. Bone marrow stromal cells from male rats were transplanted directly into the lesion of female rats that had been subjected to traumatic cortical damage (Bonilla et al., 2009). This study showed that in situ hybridization specific to the murine Sry gene can be used to confidently discriminate between donor and host cells up to two months after transplantation. Co-labeling of Neu-N or GFAP in Sry-positive cells, suggested that these cells of mesenchymal lineage possessed the capacity to undergo transdifferentiation into neuronal or glial cell types (Bonilla et al., 2009). This observation effectively illustrates the concept that in situ hybridization for the Sry gene can be used to identify transplanted cells after long periods of time regardless of differentiation into drastically different cell phenotypes. Y-Chromosome markers have been used similarly in other disease models including liver disease, skin disease, GI disease and cardiac disease (Theise et al., 2000; Deng et al., 2005; Jiang et al., 2006; Kudo et al., 2007).

Unfortunately, the use of FISH to detect Y-chromosomes in transplanted male donor cells precludes the possibility of autologous cell-based treatments, as the host must necessarily be female to differentiate host cells from male donor cells. In addition, this technique still suffers from the same histological drawbacks as other conventional histological cell tracing techniques. Namely, that any cell observation must be done post mortem and can only provide one data point in time per animal. This limitation can be quite consequential in cellular migration studies.

Lac-Z reporter

Using an intracerebral hemorrhage model (ICH) induced by intrastriatal administration of bacterial collagenase, rats received Lac-Z positive human neuronal stem cells (NSC) intravenously one day after collagenase treatment. X-gal histochemistry detected ß-galactosidase-positive transplanted hNSC. Co-labeling for cell phenotype markers such as NeuN or GFAP, revealed that the transplanted NSCs migrated specifically to the periphery of the hematomal region with a majority (~75%) subsequently differentiating into astrocytes and a smaller percentage (~10%) into neurons (Jeong et al., 2003).

Fluorescent dyes

Nuclear and cytosolic dyes

There are a host of florescent dyes available for cell labeling, each with its own set of benefits and limitations. Among them is DAPI, a simple nuclear stain, which boasts high labeling efficiency, ease of use, and low toxicity. However, lysis of dead pre-labeled cells may cause false positives for host cells in the surrounding tissue environment, as the label is released and then retained by host cells in close proximity.

Hoechst dyes are also available to stain the nuclei of transplanted cells. As Hoechst is a DNA- binding dye, it may interfere with DNA replication or transcription and, therefore, may adversely affect cell viability.

CFSE (Carboxyfluorescein succinimidyl ester) is a cytosolic protein-binding label. It forms irreversible covalent bonds with free amine groups such as those in the side chain of lysine residues and permits cell tracking by fluorescence (Hemmrich et al., 2006).

Fluorescent membrane dyes

Other types of cell tracing dyes include lipophilic carbocyanine-based dyes such as the thiol-reactive membrane dyes, CM-Dil and CM-DiO (CellTracker, Invitrogen). CM-Dil and -DiO both contain a thiol-reactive chloromethyl moiety, which reacts with membrane thiols, bonding covalently. Thus, these dyes are retained strongly and exhibit very low tendency to transfer from labeled donor cells to adjacent host cells. In addition, Dil is noted to persist through most fixation and permeabilization protocols.

CM-DiI has been used to label bone marrow fibroblasts. Successful labeling was achieved at relatively low dye concentrations (20 μM) and in a short period of time (30 min). As expected, there was a decrease in fluorescence intensity that was proportional to the number of cell divisions after staining (Ferrari et al., 2001).

CM-DiO, was used to label endogenous migratory neural stem cells residing in the adult mouse subventricular zone. CM-DiO permitted tracking of the subventricular zone NPC migration, confirming that they can and do migrate to the site of traumatic cortical injury (Salman et al., 2004). With double staining for NeuN, GFAP and Nestin, it was shown that these endogenous NPCs not only migrate to the site of cortical trauma but that they also play a primary role in glial scar formation. CM-DiO contains the same thiol-reactive chloromethyl moiety as CM-Dil, giving it the ability to form persistent, long-lasting covalent bonds to membrane thiols. Thus, this approach enabled the visualization of labeled neural stem cells up to three weeks after the initial intraventricular infusion of the dye (Salman et al., 2004).

While these dyes have a number of significant advantages including the ability for detection up to 60 days post transplantation (Ford et al., 1996) they are also plagued from a number of limitations. Like many other methods, CM dyes suffer from decreased detection with each successive mitotic cell division (Ferrari et al., 2001). This does not prohibit their use to examine single time points, but does limit their usefulness in long-term studies of proliferation. More importantly, the most significant problem with CM dyes is cytotoxicity. It has been suggested that CM-DiI has a relatively high level of toxicity (Hemmrich et al., 2006). Thus, this labeling method cannot be used for studies of stem cell survival after transplant or efficacy of stem cell-based treatments.

Fluorescent proteins

In 2009, Harting et al. reported that isolation and culture of MSC from green fluorescent protein-positive (GFP+) transgenic rodents resulted in a substantial and regular reduction in GFP expression. These studies indicated that only 50% of isolated transgenic MSC expressed GFP. It was, however, shown that differentiation of these cells did not significantly affect the GFP expression rate (Harting et al., 2009a). In light of the poor GFP expression ratio of MSC isolated and cultured from GFP+ transgenic rodents, compounded by the complexity and cost of developing and maintaining transgenic strains, vector-mediated GFP transfection is still the preferred method of GFP labeling. To this effect, Zhang et al. created the plasmid pEGFP-C2-TH (tyrosine hydroxylase) and with it has transfected cultured neuronal stem cells. Their results show that five days post-transfection, 62% of transfected cells were GFP+ (Zhang et al., 2008).

GFP transfection can be used to track engineered NPCs as they migrate through the cortex from a perilesional transplantation site to the lesion caused by a fluid percussion injury. In this case, GFP transfection allowed the detection of modified NPCs up to six weeks after transplantation (Bakshi et al., 2006).

GFP has also been successful in tracking cell migration over much larger distances. In 2008, Liu et al. used adenovirus GFP to track bone marrow stromal cell migration in an autologous transplant model after TBI in rabbits. Here it was established that bone marrow derived stem cells transplanted intrathecally via lumbar puncture will not only migrate to the brain but will preferentially localize around injured tissue (Liu et al., 2008).

A similar fluorescent protein approach was employed by Hung et al. in 2010 to generate an immortalize cord blood-derived MSC line that stably expressed red fluorescent protein (RFP). Here the investigators injected RFP-MSC derived from cord blood into the lateral ventricle of rats that received experimentally induced TBI 3 days earlier. 14-days after cell delivery, RFP-MSC were visualized in the region of injury (Hung et al., 2010).

Quantum dots

Recent advances in fluorescent nanocrystal technology have yielded commercially available fluorescent quantum dots (Qdots) such as QTracker 655 (Invitrogen). Qdots, as opposed to conventional cell labels and dyes, have the major added benefit of strong, persistent and quench-resistant fluorescence. In addition to this, Qdot cell loading protocols typically achieve the desired delivery in very little time. This method has been used in tracking rat MSC, which were intravenously delivered subsequent to traumatic brain injury (Harting et al., 2009b). The bright, stable Qdot fluorescence facilitated the identification of labeled cells in various

tissue types including pulmonary and brain tissue. Although Qdot cell labeling suffers from the same limitation of label-dilution after subsequent rounds of mitotic division, the distinct, indefatigable emission from even a single Qdot is detectable.

Magnetic resonance imaging

There are several available *in vivo* imaging modalities that can potentially provide longitudinal cell tracking data. The ability to generate longitudinal, real-time data is primarily beneficial because it allows the researcher to obtain data from multiple time-points in the same animal as opposed to conventional, histological cell tracking techniques, which necessarily require the sacrifice of an animal to obtain data from only a single time-point. Immediately, this ability overcomes the major shortcoming that is common to all previously discussed cell tracking methods. Such imaging modalities include: Computed Tomography (CT); Positron Emission Tomography (PET); Single Photo Emission Computed Tomography (SPECT) and Magnetic Resonance Imaging (MRI).

Of these imaging modalities, MRI offers the highest spatial resolution and thus, best lends itself to cellular imaging. It has been demonstrated that MRI has the capability of detecting even single labeled cells after transplantation (Heyn et al., 2006; Shapiro et al., 2006a). MRI has the added benefit of not exposing patients or test subjects to ionizing radiation and is also beneficial in clinical tracking of cell transplants in that it can provide accurate high-resolution three-dimensional anatomical information as well as relevant types of pathological information such as the extent of inflammation, edema and angiogenesis. This is done by way of volumetrics and diffusion weighted imaging (Anderson et al., 2005; Iwanami et al., 2005; Schepkin et al., 2012).

Magnetic resonance was first employed by Modo et al. in 2002 to track transplanted stem cells in a rat model of ischemic injury. Since then, MRI has been used to successfully track transplanted stem cells in a variety of disease models including: traumatic brain injury (Li et al., 2011), ischemic brain injury (Rice et al., 2007) and cardiac disease models (Amsalem et al., 2007; Ebert et al., 2007). Recently, MRI has been used to track an endogenous, migratory population of NPCs, which arise in the subventricular zone (SVZ) of the adult mammalian brain and migrate to the olfactory bulb (Shapiro et al., 2006b). These data show that MRI not only has viable applications in tracking exogenous cell transplants but can also be used in the study of native cell migration.

Iron-based contrast agents for MRI

For transplanted cells to be distinguished from surrounding host cells, they must be pre-labeled with an MRI-sensitive contrast agent. There are a variety of MR-active particles, which can be applied to cell populations by various loading protocols. Different types of MRI contrast agents can produce either a "positive" MR signal or a "negative" signal; a signal void. The two most common types of MR contrast agents being utilized are iron oxide- and gadolinium-based particles. Due to their use in the majority of MR cell tracking studies, we will focus on iron-based MR contrast agents.

Due to their prominent T2* effects, iron nanoparticles have been widely adopted in magnetic resonance cellular imaging (Bulte and Kraitchman, 2004; Shapiro et al., 2004, 2006a,b). Iron oxide nanoparticles can be categorized according to size. Superparamagnetic Iron Oxide particles (SPIO) range in size from 50-200nm in diameter. Ultra-small Super Paramagnetic Iron Oxide particles (USPIO) measure less than 50nm in diameter and micron-sized paramagnetic particles (MPIO) measure on the order of 1 μm in diameter. These particles produce substantial local magnetic field inhomogeneities, which results in diminished T2 and T2* relaxation times. On MRI, this appears as a signal void, shown as hypointense (black) contrast.

Iron oxide nanoparticles are generally consistent in their construction. Typically, they are comprised of a mixed-valence (Fe^{2+} and Fe^{3+}) iron core with varying chemical coatings. Common nanoparticle coatings include dextran, carboxydextran, silica (Matuszewski et al., 2005; Kim et al., 2007), polyethylene glycol (Basly et al., 2010), and polystyrene (Seymour et al., 1991). Nanoparticle coating helps limit aggregation and cellular toxicity (Zhang et al., 2009) and can also be manipulated to optimize MRI contrast properties (Matuszewski et al., 2005).

Due to their large size and higher iron content, MPIOs are the most detectable of the iron-based MR contrast agents (Slotkin et al., 2007). However, most MPIOs are coated with polystyrene, which lacks the appealing quality of being biodegradable. Furthermore, currently available MPIOs cannot be acquired with reliable sterility, which practically precludes the option of clinical MPIO usage.

Conversely, iron labels like Feridex (Berlex Pharmaceuticals, USA) or Endorem (Guerbet, France) are dextran-coated SPIOs with particle sizes ranging from 50-180nm in diameter. The dextran coating makes them very attractive labeling options for live-animal and clinical studies, as the particles can be biochemically degraded via regular iron metabolism mechanisms (Ferrucci et al., 1990). Although Feridex was FDA-approved in 1996 for clinical use as liver MR contrast agent, its production was discontinued in 2008 and is thus no longer commercially available. Endorem has similarly been discontinued. There are, however, other types of iron nanoparticles available, although none are currently approved for clinical use. For example, fluorescent encapsulated magnetic microspheres with a carboxyl coating and a choice of fluorescence are available commercially (Bangs Labs, Fishers, Indiana).

Syková and Jendelová have been successful in showing that the use of Endorem iron oxide nanoparticles can be used effectively to track the migration of transplanted mouse embryonic stem cells (ESC), human MSC and rat MSC. In this case, it was shown that these cells, administered either intravenously or by direct transplant , could be visualized in vivo, and their migration pattern and tendency to migrate to sites of injury could be traced using MRI over extended periods in real time (Syková and Jendelová, 2006).

In the context of traumatic brain injury, it has been shown that systemically administered, SPIO-labeled human MSC can be visualized by MRI as they preferentially accumulate in penumbral region up to six weeks post-TBI (Li et al., 2011). This provides an excellent example of the viability of MRI in long term *in vivo* cell tracking studies. Alternatively, a new highly sensitive magnetite nanoparticle has been constructed and appears to have promising applications in cellular MR imaging. This superparamagnetic iron oxide nanoparticle (SPION) was coupled to 2-aminoethyl-trimethyl ammonium (TMA). This TMA-SPION possesses a strong positive charge and a hydrodynamic size of 101nm. Among its benefits are long-term stability in aqueous solution, 4.4x greater T2 relaxivity than Feridex, short labeling time and no need for transfection agents. The authors showed that this iron-based nanoparticle was effective in tracking hMSC migration in a photochemically induced mouse brain ischemia model (Kim et al., 2011).

Ferritin and MRI

As cellular and molecular imaging techniques, like MRI, develop, it becomes increasingly important to collect more than just the spatial and temporal information about labeled cell populations. Information pertaining to the activity of tagged cells requires a reporter. Ferritin, a ubiquitous iron-sequestering metalloprotein with ferroxidase activity, was first suggested for use as an MRI reporter gene. It was shown to be an effective transgene with stable *in vivo* expression in glioma tumors (Cohen et al., 2005) and *in vitro* in adenocarcinoma A549 cells (Genove et al., 2005). In both instances, over-expression of Ferritin, which becomes superparamagnetic upon iron binding, generated sufficient local field inhomogeneities to be visualized on T2- and T2*-weighted images. Since then, Ferritin has proven successful as an MRI reporter in transplanted mouse neural stem cells (Deans et al., 2006), separate glioma lines (Ono et al., 2009), and swine cardiac progenitor cells (Campan et al., 2011).

Ferritin has also been optimized for enhanced iron loading and transverse MR relaxation times; and therefore, enhanced visualization. It was shown that a light and heavy chain chimeric ferritin is capable of storing more biogenic iron than the wild type or homopolymeric analogs; ultimately yielding enhanced contrast in MR images (Iordanova et al., 2010). This approach has the additional advantage of being potentially controlled by tetracycline or similar regulation in vivo (Cohen et al., 2005).

Although MRI has the unique benefit of allowing non-invasive *in vivo* cell tracking, it also suffers from some shortcomings different from those of histologically-based cell tracking techniques. While MRI methods can track the location of cells over time, they are incapable of identifying cell phenotype. In stem cell fate-oriented studies, this information is vital. To phenotype a transplanted cell, it is still necessary to employ standard immunohistochemical protocols. Furthermore, given the inability of MRI to properly address the question of cell phenotype, it is impossible to determine whether the cell label has been retained by the original transplanted cell or if it has been released and subsequently endocytosed by another cell type; for example, in an immune response. While these challenges will clearly need to be addressed by future research, the potential for the use of non-invasive methods such as MRI to track stem cell migration and survival and their potential clinical applications clearly warrant further investigation.

Conclusions

The conventional cell tracking methods discussed here suffer from a common set of limitations. For most, these include reduced proliferation and survival after labeling. This is an artifact of labeling that can have serious ramifications in determining the efficacy of stem cell-based therapies. Another major restriction, which is a hindrance to all conventional cell tracing procedures is that cell migration information can only be determined upon examination of tissue *ex vivo*. In a pre-clinical context, this deficiency prevents repeated measures in the same animal and the collection of longitudinal data. In a clinical setting, these approaches cannot be used outside of a post-mortem analysis.

While MRI is subject to its own, but different, limitations, it has the ability to circumvent the most detrimental shortcomings of standard cell labeling and tracking systems. While MRI has not yet supplanted these strategies, this review has shown that it can serve as a powerful adjunct to other cell labeling techniques and has the potential to become a vital investigative tool for clinical monitoring of stem cell-based therapies.

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