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Stem and Progenitor Cells for Neurological Repair: Minor Issues, Major Hurdles, and Exciting Opportunities for Paracrine-Based Therapeutics

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Abstract

The transplantation of cultured stem and progenitor cells is a key element in the rapidly growing field of regenerative medicine. Based on their ability to rescue and/or repair injured tissue and partially restore organ function, multiple types of stem/progenitor cells have already entered into clinical trials. However, despite several decades of intense research, the goal to apply culture-expanded stem/progenitor cells in a manner that can effectively replace cells after injury has yet to be realized. Many sources of potentially useful cells are available, but something is clearly missing. In addition, recent studies suggest that paracrine effects of secreted or released factors are responsible for most of the benefits observed after cell transplantation, rather than direct cell replacement. These data call into question the need for cell transplantation for many types of therapy, in particular for acute injuries such as myocardial infarction and stroke. In this review, we examine current progress in the area of cell transplantation and minor issues and major hurdles regarding the clinical application of different cell types. We discuss the "paracrine hypothesis" for the action of transplanted stem/progenitor cells as an opportunity to identify defined combinations of biomolecules to rescue and/or repair tissues after injury. Although many of the concepts in this review will apply to multiple injury/repair systems, we will focus primarily on stem/progenitor cell-based treatments for neurological disorders and stroke.

INTRODUCTION

It is widely hoped that transplantation of stem/progenitor cells will provide effective therapies for many neurological diseases and injuries such as Parkinson's disease, Alzheimer's disease, Huntington's Disease, amyloid lateral sclerosis, spinal cord injury, and stroke [Lindvall and Kokaia, 2006; Martino and Pluchino, 2006; Gogel et al., 2010; Kiskinis and Eggan, 2010]. Numerous encouraging animal studies have shown that stem or progenitor cell treatments can rescue some degree of neurological function after injury. Moreover, a variety of clinical trials have been performed and others are currently ongoing [Prockop and Olson, 2007; Mazzini et al., 2008; Gogel et al., 2010; Lee et al., 2010].

Stem cells are specialized cells that self-renew and are typically bipotent or multipotent such as adult stem cells and fetal stem cells, or are pluripotent such as embryonic stem cells (ES cells) and induced pluripotent stem cells (iPS cells). Progenitor cells lie downstream of stem cells in terms of lineage and often possess reduced capacity for self-renewal and reduced potency for differentiation relative to stem cells. Stem/progenitor cells from a variety of

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sources have rescued injured tissue and improved functional recovery when used to treat models of stroke, including ES cells, iPS cells, neural stem/progenitor cells (NSC/NPC), and bone marrow-derived non-hematopoietic multipotent stromal cells (BMSC) [Chen et al., 2001; Haas et al., 2005; Bliss et al., 2007; Bacigaluppi et al., 2009; Hicks et al., 2009; Kawai et al., 2010]. With all the different choices, which stem or progenitor cells should be used for particular treatments? Which are ready for clinical application? The first aim of this review will be to examine some of the pros and cons surrounding currently available types of stem/progenitor cells and their relative potential for use in tissue rescue, tissue repair, and cell replacement strategies.

We have made rapid and extensive progress in optimizing the isolation and culture of many types of stem/progenitor cells, but our ability to use them to replace diseased or necrotic tissue after injury has progressed at a far slower pace. Notably, few studies have demonstrated direct evidence of cell replacement in injury or disease models that clearly explains the benefits observed after cell therapy. Many positive outcomes after cell therapy appear to be attributed to rescue of pre-existing tissue rather than repair or cell replacement per se. The paracrine action of growth factors, cytokines, and hormones that are secreted or released by transplanted cells has been shown to provide most of the benefits after stem/progenitor cell administration. This can be seen as a problem, since for many years we missed paracrine activity as a principal mechanism in cell therapy and the paracrine mechanism(s) may be complicated. Alternatively, the situation can be viewed as an exciting opportunity to learn from the paracrine biology and biochemistry of stem/progenitor cells and to identify the key secreted factors that conferred benefit in the past. With detailed knowledge regarding the compositions of factors secreted by reparative cells, an acute rescue of pre-existing tissue or stimulation of endogenous repair processes may be possible with the administration of defined combinations of proteins, peptides, and molecules rather than cells. The second aim of this review will be to discuss the paracrine biology and biochemistry of stem and progenitor cells and how we may learn to provide powerful paracrine-based therapeutics to rescue and/or repair injured tissues.

Stem/progenitor cells with promise to treat neurological diseases and stroke

ES cells—Human ES cells are derived from the inner cell mass of blastocyst-stage embryos [Thomson et al., 1998]. ES cells are characterized by an extensive self-renewal capacity and pluripotency to differentiate into all somatic cell types. Due to their pluripotency, human ES cells have potential for cell replacement therapy in a wide variety of diseases and tissue injuries. The research community has made rapid progress in learning how to drive the differentiation of specific cell lineages or cell types from undifferentiated ES cells. ES cells from rodents have been used to generate dopaminergic neurons to treat models of Parkinson's Disease [Kim et al., 2002; Rodriguez-Gomez et al., 2007], provide progenitors that promote recovery after spinal cord injury [McDonald et al., 1999], and to replace photoreceptors to restore vision [Lamba et al., 2009]. Transplantation of human ES cell neural derivatives into a rodent model was shown to improve functional outcome after stroke [Hicks et al., 2009]. Furthermore, based on their extensive growth properties, ES cells are readily amenable to gene correction through the use of viruses, zinc finger nucleases, or homologous recombination.

Although many researchers are excited about the clinical application of ES cells, there are several problematic issues surrounding human ES cells that include: ethical/political issues (i.e. destruction of human embryos), tumor formation, immune rejection, and the relative "age" of the cell derivatives to be grafted. For example, it has been largely overlooked that most ES cell derivatives to be grafted will be fetal in nature. Therefore, grafted ES cell derivatives may lack important functional characteristics of adult cells [Xi et al., 2010].

In 2010, US federal funding for human ES cell research was temporally disrupted due to ethical/political issues. Funding was restored, but the situation underscored that these issues are not entirely resolved and may become important again as ES cell technology is used in the clinic. It is well known that ES cells form teratomas (developmental tumors) following transplantation to rodents [Knoepfler, 2009]. However, it is not known how tumors arise from ES cells or how to prevent tumor formation after transplantation. This lack of knowledge has, in part, delayed the use of ES cells or their derivatives to treat humans. Although some investigators still experiment with the administration of undifferentiated ES cells, most have moved to the purification of defined cell populations that differentiate into particular cell lineages or cell types. Interestingly, the host environment may play an important role in whether ES cells or their derivatives produce tumors or not. Erdö et al. reported that undifferentiated and pre-differentiated murine ES cells did not form tumors when transplanted into rats with stroke, whereas transplantation of murine ES cells or their derivatives into a homologous mouse stroke model resulted in large cerebral tumors [Erdö et al., 2003]. A different study identified the immune response as an important determinant of tumor formation by ES cells and their derivatives in xenogeneic versus syngeneic or allogeneic transplants [Dressel et al., 2008].

In July 2010, the US Food and Drug Administration (FDA) approved phase I clinical trials of human ES cell-derived oligodendrocyte progenitor cells for treatment of spinal cord injury [Keirstead et al., 2005]. Despite the highly publicized milestone for the first clinical trial involving human ES cells, safety concerns remain and much of the scientific community still questions whether it was time yet to initiate clinical trials with ES cells or their derivatives. Seminatore et al. recently demonstrated that ES cell-derived neural progenitor cells were influenced to become hyper-proliferative when exposed to factors produced in the ischemic environment of stroke [Seminatore et al., 2010]. This study indicates that the differentiation state of ES cell derivatives must be carefully monitored to prevent tumor formation in the injury environment. Furthermore, the responses of transplanted fetal stage cell derivatives that are actively engaged in a developmental program may not be the same as those of adult stem/progenitor cells that participate in tissue repair after injury but do not form tumors. Many controlled animal studies should be conducted if ES cell derivatives will be used to provide safe and effective therapeutics for patients.

iPS cells—iPS cells were first derived by the Yamanaka group in 2006 [Takahashi and Yamanaka, 2006; Takahashi et al., 2007]. They re-programmed mouse and human fibroblasts into pluripotent embryonic stem-like cells through the virally-induced expression of four transcription factors: Sox2, c-Myc, Klf4 and Oct3/4. Since then, many groups have created iPS cells using diverse cell types, different factors and various methods [Kiskinis and Eggan, 2010; Takahashi, 2010]. Although iPS cells appear to be very similar to ES cells, recent data indicate that they also possess some differences [Kim et al., 2010]. Investigators are now actively determining the degree of epigenetic memory and determinants of clonal variability in iPS cell lines. These variables can influence the differentiation and function of iPS cell derivatives.

The iPS cells have gained a wide following because they possess most if not all of the key properties of ES cells but avoid the ethical/political issues surrounding embryo destruction. Similar to ES cells, the potential for large scale expansion of iPS cells make them amenable to genetic correction. Moreover, it is possible to derive patient-specific iPS cells that should avoid immune rejection [Kiskinis and Eggan, 2010]. Many academic and commercial groups are taking advantage of this aspect of iPS cell technology in another way, by deriving a wide variety of lines from patients with heritable genetic diseases [Dimos et al., 2008]. It is felt that these lines will provide invaluable disease models for the identification of drug

targets and for screening of chemical libraries for bioactive compounds and treatments [Kiskinis and Eggan, 2010].

Investigators have used iPS cells to treat CNS injuries such as spinal cord injury and stroke in rodents [Kawai et al., 2010; Tsuji et al., 2010]. However, in both cases, tumor formation from iPS cells was observed. In the case of spinal cord injury, tumor formation was found to prevent functional repair. Several studies suggest that iPS cells may have an even greater propensity to form tumors than ES cells, although this may depend on the method used for derivation. Accordingly, the standardized isolation of non-tumor forming iPS cell derivatives should be one of the first steps prior to their application in cellular therapy [Tsuji et al., 2010]. Similar to ES cells, detailed and stringent pre-clinical studies in animal models should preclude any clinical application of iPS cell derivatives.

Fetal neural stem cells (NSC)—Because of the invasive nature of obtaining autologous adult human NSC, many investigations have focused instead on fetal NSC as an expandable source for neural cells. Fetal NSC are derived from human fetal brains and are capable of differentiating into neurons, astrocytes and oligodendrocytes [Lindvall and Kokaia, 2006]. They are generally isolated from aborted material and are less ethically/politically controversial than ES cells. Historically, fetal NSC have been considered safer than human ES cells in terms of tumor formation after transplantation. Neural cells of fetal origin have been applied with mixed results to treat both Parkinson's Disease patients as well as in Huntington's Disease patients [Lindvall and Kokaia, 2006; Cicchetti et al., 2009].

The source of the fetal cells to be transplanted and the transplant environment itself are likely to be critical determinants for clinical outcomes in therapies using fetal cells. For example, problems were reported in 2009 for a boy with ataxia telangiectasia that was treated with human fetal NSC in Russia at 9, 10 and 12 years of age. Tragically, 4 years after the first transplantation, tumors were found in the brain and spinal cord of the boy [Amariglio et al., 2009]. This incident illustrated that human fetal NSC require further evaluation as a potential therapeutic. It is not known whether the environment of transplantation, the cell type transplanted or both played a role in tumor formation. Similar to ES cell and iPS cell derivatives, human fetal NSC should be carefully screened for tumor formation prior to administration. Simple tumor formation assays in healthy immunodeficient mice will not be informative enough. Prior to future clinical applications, cell derivatives from ES cells, iPS cells, and fetal NSC should all be carefully tested for their responses to specific injury environments that mimic the injury environments in patients that will receive cells. These types of pre-clinical studies may help to identify populations of cultured cells that should be avoided or patients that should probably not receive developmentally-related cell therapies.

BMSC—The first non-hematopoietic bone marrow-derived multipotent stromal cells (BMSC), a progenitor cell type commonly referred to as "mesenchymal stem cells", were reported in 1976. Friedenstein et al. described clonal, plastic adherent cells from bone marrow capable of differentiating into osteoblasts, adipocytes, and chondrocytes [Friedenstein et al., 1976; Friedenstein et al., 1987]. BMSC are attractive for cell therapy because they can be easily obtained from human bone marrow aspirates, can be rapidly expanded in culture, and can be used autologously. Importantly, BMSC do not form tumors after transplantation [Phinney and Prockop, 2007]. Several clinical trials, primarily to treat acute myocardial infarction (heart attack), have begun with BMSC based on their safety in dose escalation studies in humans and lack of tumor formation [Prockop and Olson, 2007]. A recent long-term follow-up for a clinical trial in stroke with intravenous BMSC treatment reported improved survival statistics for patients that received BMSC compared with controls in addition to other positive outcomes [Lee et al., 2010].

Many reports have shown that BMSC express proteins commonly associated with neurons. However, direct functional evidence of neuronal differentiation from normal non-genetically modified BMSC, in the absence of cell fusion, is lacking *ex vivo* and *in vivo* [Phinney and Prockop, 2007]. Whereas BMSC appear to be useful to replace or repair bone, tendon, or other mesenchymal and connective tissue types, they are unlikely to be an effective source for replacement of neural cells. Interestingly, despite their lack of neuronal differentiation, transplantation of BMSC can rescue injured CNS tissue and improve function. Positive effects following intravenous or intra-arterial infusion of BMSC after stroke have been reported in rats, mice and humans, even without significant levels of BMSC engraftment [Chen et al., 2001; Bakondi and Shimada et al., 2009; Lee et al., 2010].

Due to low levels of engraftment and lack of neuronal differentiation, secreted factors from transplanted cells are thought to play an important role in functional recovery after stroke and BMSC treatment. This concept is often referred to as the “paracrine hypothesis” for BMSC action. Paracrine effects appear to be relevant for most if not all injury models in which BMSC treatment is effective and applies also to treatments with other types of adult stem/progenitor cells [Gnecchi et al., 2008]. Notably, the paracrine activity of injected ES cells also has been shown to ameliorate ischemic tissue injury [Fatma et al., 2010].

Subpopulations of BMSC—Because the profiles of factors secreted by subpopulations of progenitor cells may differ based on their roles in vivo, lineage progression, and differential responses to environmental stimuli, we recently began to isolate subpopulations of adult stem/progenitor cells by specific cell surface epitopes to investigate their relative ability to protect cells after tissue injury. Such an analysis can be useful in terms of identifying either cells or secreted ligands that provide rescue and/or repair of injured tissue. BMSC are commonly isolated by density gradient centrifugation to obtain total bone marrow mononuclear cells and then by simple adherence to tissue culture plastic. BMSC isolated in this manner are heterogenous in nature, potentially contaminated by other cell types, and difficult to standardize; this may lead to variability in treatment effects. When isolated by simple plastic adherence, BMSC cultures generated from left and right ileac crest aspirates of the same patient can vary in their growth or differentiation potential. With the aim of developing standardized cell-based therapies, we and others have isolated BMSC from bone marrow by fluorescent-activated cell sorting (FACS) or magnetic activated cell sorting (MACS). Several groups have described cell surface epitopes that prospectively isolate BMSC including CD49b, CD73, CD90, CD105, CD130, CD140b, CD146, CD200, CD271, CD340, CD349, integrin alphaV/beta5, and STRO-1 [Gronthos et al., 1994; Quirici et al., 2002; Buhring et al., 2007; Sacchetti et al., 2007; Delorme et al., 2008]. Notably, the different epitopes isolate BMSC-like cells with varying degrees of clone forming efficiency, growth potential, and differentiation potential. Sacchetti et al isolated human BMSC based on CD146 (MCAM) expression and identified osteoprogenitor cells capable of self-renewal and of generating ectopic hematopoietic microenvironments in immunodeficient mice [Sacchetti et al., 2007]. We reported that CD133 (Prominin-1) could be used to isolate human bone marrow stem/progenitor cells that gave rise to BMSC in culture. Similarly, the derived CD133BMSC also produced ectopic hematopoietic environments following transplantation to immunodeficient mice [Bakondi and Spees, 2010]. Further research is needed to fully characterize BMSC that are isolated by different cell surface proteins. Some of the epitopes listed above may be useful to isolate the actual non-hematopoietic stem cell from bone marrow, while others may isolate downstream progenitor cells or subpopulations of progenitor cells.

Paracrine effects of BMSC subpopulations and treatment of stroke

Our laboratory recently compared human BMSC isolated by virtue of cell surface expression of CD133 (CD133BMSC) or CD271 (p75 low affinity nerve growth factor receptor, p75BMSC) [Bakondi and Shimada et al., 2009]. We found that these BMSC subpopulations differed from each other and from typical BMSC isolated by simple plastic adherence in terms of their secreted factors, secretion responses during hypoxia exposure, and the relative ability of their compositions of secreted factors to provide a treatment for stroke injury [Bakondi and Shimada et al., 2009]. We first compared arterial infusion of CD133BMSC cells with infusion of phosphate buffered saline (cell vehicle control) and infusion of cell- and serum-free conditioned medium (CdM, 40-fold concentrated). We treated immunodeficient mice 1 day after distal middle cerebral artery ligation and determined infarct volume 2 days after treatment. In support of the paracrine hypothesis for BMSC action, the CD133BMSC CdM significantly reduced infarct volume after stroke. Furthermore, although the injection of CD133BMSC (cells) also significantly reduced infarct volume compared with PBS injection, it was not as effective as the CdM injection. This observation is important because it indicates that BMSC do not necessarily need to be exposed to the stroke environment in vivo to express and release new factors. Rather, the process of removing their nutrients and culturing them in serum-free medium for 2 days provided an adequate stimulus to allow us to collect a therapeutic composition of secreted neuro- and/or vaso-protective factors. There may be a variety of different culture manipulations that can be used to “prime” BMSC before cell administration, to generate powerful CdM compositions for treatment, or to screen for novel combinations of therapeutic proteins, peptides, and hormones.

Because CD133BMSC CdM was more effective than CD133BMSC (cells) to treat stroke, we then compared CD133BMSC CdM to CdMs from p75BMSC (a different BMSC subpopulation), typical human BMSC, and human dermal fibroblasts (control). All of the CdMs for comparison were matched for protein concentration prior to treatment. In agreement with our data indicating differences in the levels of several secreted proteins by ELISA, the p75BMSC CdM did not protect against stroke. Nor did the CdM generated from dermal fibroblasts. CdM from typical BMSC significantly reduced infarct volume after stroke, but was not as effective as CD133BMSC CdM. Therefore, CD133 identifies a subpopulation of human bone marrow stem/progenitor cells with an enhanced capacity to treat stroke injury based on their repertoire of secreted proteins and peptides [Bakondi et al., 2009]. It is now of great interest to identify the protein and peptide components in CD133BMSC CdM that provided neuro- and/or vaso-protection after stroke [Bakondi et al., 2010].

Future Directions for cell therapy and paracrine-based therapeutics

To effectively use developmentally-related cell types such as ES cells, iPS cells, fetal NSC and their derivatives for repair of neurological tissues, we will clearly need to understand why they form tumors and how to isolate non-tumor forming cell derivatives. It will also be critical to understand environmental factors that initiate or promote tumor formation in normal and injured tissues. Aside the immediate issue of tumor formation, we need also to determine whether various stem cell treatments have any long-term negative side effects. For cell replacement using either developmentally-related stem/progenitor cells or adult stem/progenitor cells, we need to improve our knowledge of injury environments in order to facilitate engraftment and differentiation of transplanted cells. Regardless of the particular stem or progenitor cell type being transplanted, almost all studies to date share one thing in common: the vast majority of transplanted cells die shortly after administration. For effective cell replacement using cultured cells, that often lose important homing and adhesion receptors during expansion, it would probably be helpful to first understand

mechanisms of tissue rescue and/or repair by endogenous reparative cells in adults. For example, we could be treating injury/disease models with the proper cells, but the cells are in the wrong "cell state". We may be losing many transplanted cells because they are lifted from culture in S-phase but require a different stage of the cell cycle to survive transplantation.

To accurately predict clinical outcomes, we need to better define how stem cell treatments work for each disease or injury system. For example, in the treatment of stroke, different studies have shown that stem cell treatments act to increase cell survival, increase angiogenesis, improve synapse formation, reduce inflammatory responses, and decrease glial scarring [Lindvall and Kokaia, 2004; Martino and Pluchino, 2006; Bacigaluppi et al., 2009]. While all of these effects may occur simultaneously, some mechanisms may be more important than others. Also, some mechanisms may be direct and some may be indirect.

Understanding the paracrine biology of reparative cells should lead to powerful new combinatorial therapies. Importantly, although concentrated CdM from stem/progenitor cells may act as an effective therapeutic, the FDA will likely prefer a standardized regimen of defined factors that can be produced and quality-controlled. There is already a history of administering growth factors to treat neurological injuries such as stroke [reviewed in Ren and Finklestein, 2005; Greenberg and Jin, 2006; Lanfranconi et al., 2009]. Most treatment paradigms have used high dose administrations of single factors or 2 factor combinations that include: IGF-1, EPO, NGF, BDNF, SCF, GCSF, HB-EGF, EGF, SDF-1, bFGF, and/or VEGF. However, many cytoprotective factors, like bFGF or VEGF, are not effective at high doses (e.g. bFGF becomes toxic at high doses and VEGF promotes blood vessel leakage). This is probably one reason why several clinical trials that administered 1 or 2 growth factors did not yield effective treatments for stroke. Another is the difficulty in delivering factors in a localized manner so as to avoid off-target effects. For many CNS injuries, there is also difficulty in treatment delivery and crossing intrinsic barriers such as the blood brain barrier (BBB). Encouraging developments in the intranasal delivery of growth factors to treat stroke may provide one way to circumvent the BBB issue [Hanson and Frey, 2008].

Multi-factorial processes such as tissue rescue and repair after injury may not be possible to manipulate efficiently by administering a single protein, peptide, antibody, or drug. Tissues are complex and composed of multiple cell types; they present multiple targets. To effectively use recombinant proteins, peptides, and other molecules for tissue rescue/repair, we will probably need to move away from traditional pharmacological systems based on 1 or 2 growth factors or molecules. Instead, we need to identify relevant combinations of factors and their relative concentrations in order to mimic how endogenous reparative cells function in vivo. Imagine bone marrow progenitor cells that circulate, home to tissue injury on chemokine gradients, and then respond in a paracrine fashion once they arrive at the site of injury. They are unlikely to extravasate through blood vessel walls, arrive on the injury scene and, by analogy, play a single loud note to initiate rescue and repair. Rather, they more likely to play a song; secreting multiple factors, in the right order and in the right concentrations to effectively orchestrate a variety of rescue and repair processes. The effects may improve cell survival, increase angiogenesis, reduce inflammation, and mobilize endogenous reparative cells-all simultaneously. This type of repair system is powerful because it provides positive effects with few negative side effects by using low concentrations of multiple factors. In contrast, treatment of injured tissues with high concentrations of single factors will probably be less effective and prone to a larger number of side effects.

For effective regenerative medicine after acute injuries such as stroke, we should carefully study the paracrine action of stem/progenitor cells to learn the "codes" of proteins, peptides,

and hormones that they use to preserve injured tissue and initiate endogenous repair processes. To rescue injured tissues, it may be possible to identify 5 to 10 factors per tissue type that will be effective when used in the correct combination, even at relatively low concentrations. Looking ahead, it makes sense that a combinatorial therapy with defined factors would provide the first treatment regimen for an acute injury like stroke. The first treatment could then be followed by administration of adult stem/progenitor cells, fetal cells, ES cell derivatives or iPS cell derivatives to replace those cells not rescued by the first treatment regimen.

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