Introduction

Despite recent advances in neonatal intensive care medicine, neonatal brain injury resulting from intraventricular hemorrhage (IVH), hypoxic-ischemic encephalopathy (HIE) or neonatal stroke remains a major cause of neonatal mortality and neurologic morbidities in survivors [1–3]. Currently, few effective therapies are available to ameliorate brain injury resulting from these disorders. Therefore, the development of new, safe and effective therapies to improve the prognosis of these neurologic disorders is an urgent requirement.

Exogenous administration of stem cells significantly attenuates brain injury in the newborn animal models of IVH [4, 5], HIE [6] and neonatal stroke [7]. Furthermore, phase I clinical trials providing autologous umbilical cord blood (UCB) cells to neonates with HIE [8] or human UCB-derived mesenchymal stem cells (MSC) to neonates with bronchopulmonary dysplasia [9] have shown the treatments to be safe, feasible and potentially efficacious. Taken together these findings suggest that exogenous stem cell transplantation might be a promising new therapy for treating neonatal brain injury resulting from IVH, HIE or neonatal stroke. This review summarizes recent advances in stem cell research for treating neonatal brain injury due to intraventricular hemorrhage or hypoxic-ischemic encephalopathy with a particular focus on preclinical data, covering important issues for clinical translation such as optimal cell type, route, dose and timing of stem cell therapy, and translation of these preclinical results into a clinical trial.

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Key Words
Mesenchymal stem cells · Cell transplantation · Infant newborn · Intraventricular hemorrhage · Hypoxic-ischemic encephalopathy · Neonatal stroke

Abstract

Despite recent advances in neonatal intensive care medicine, neonatal brain injury resulting from intraventricular hemorrhage or hypoxic-ischemic encephalopathy remains a major cause of neonatal mortality and neurologic morbidities in survivors. Several studies have indicated that stem cell therapy is a promising novel therapy for neonatal brain injury resulting from these disorders. This review summarizes recent advances in stem cell research for treating neonatal brain injury due to intraventricular hemorrhage or hypoxic-ischemic encephalopathy with a particular focus on preclinical data, covering important issues for clinical translation such as optimal cell type, route, dose and timing of stem cell therapy, and translation of these preclinical results into a clinical trial.

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Stem Cells for Neonatal Brain Disorders

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on preclinical data relevant to issues essential for clinical translation such as mechanism of action, optimal cell type, route, dose and timing of stem cell transplantation, as well as successful translation of these preclinical results into a phase I clinical trial.

Preclinical Data

Therapeutic Potential of Stem Cell Transplantation for Neonatal Brain Injury

Intraventricular Hemorrhage

IVH is a serious complication of prematurity and over 50% of infants with severe IVH (grade ≥3) die or develop posthemorrhagic hydrocephalus (PHH) requiring shunt surgery in up to 70% of cases [10, 11]. Developing an appropriate animal model to simulate clinical IVH in preterm infants is very important for delineating its pathophysiologic mechanism and testing the efficacy of any potential new treatment. Therefore, we developed a newborn rat pup model of severe IVH by injecting 100 μl of dam blood into each ventricle (total 200 μl) using a stereotaxic frame at postnatal day 4 and confirmed the development of progressive PHH in 85% of the animals on follow-up brain magnetic resonance imaging (MRI) up to postnatal day 32 [4]. Therapeutic efficacy of human UCB-derived MSC transplantation has been tested in the severe IVH-induced newborn rat model and this study demonstrated that MSC transplantation significantly attenuated development of PHH, brain injury and impaired behavioral tests after severe IVH [4]. These results suggest that stem cell transplantation shows promise as a novel therapy for severe IVH.

Neonatal Stroke

Neonatal stroke, usually occurring from middle cerebral artery occlusion (MCAO), is a serious perinatal brain injury causing significant mortality and neurologic morbidity with few effective treatments [12]. Although the neuroprotective effects of exogenously administered MSC have been reported in adult stroke models of MCAO [13, 14], these beneficial results obtained in adults could not be directly extrapolated to neonatal medicine due to the dramatic differences in the maturational stage and pathophysiology of neonatal and adult brains, and need to be confirmed in the newborn animal model. We thus tested the therapeutic efficacy of human UCB-derived MSC transplantation in attenuating severe brain injury involving >50% of the ipsilateral hemisphere induced by permanent MCAO in newborn rats at postnatal day 10. We observed significant improvements in the size of brain infarct volume measured by MRI, impaired functional tests, and increased apoptosis and astroglisis in the penumbra by MSC transplantation at 6 h after MCAO [7]. These findings suggest that MSC might be a novel therapeutic candidate for severe neonatal brain injury due to neonatal stroke. Moreover, because of similarities in their pathophysiology, our data also suggest that transplantation of MSC effective for neonatal stroke could be applied to severe neonatal HIE.

Hypoxic-Ischemic Encephalopathy

Although hypothermia is the only clinically available treatment for neonatal HIE, it is not very effective – especially in severe cases [15, 16]. Therefore, additional treatments besides hypothermia that maximize neuroprotection and improve prognosis of severe neonatal HIE are urgently needed. Recently, we demonstrated that combined treatment of human UCB-derived MSC transplantation and hypothermia in severe HIE involving >50% of the ipsilateral hemisphere volume better attenuated severe HIE-induced brain injuries, such as progressively increased brain infarction, cytokine levels, apoptotic cells, microgliosis and astrocytosis, and impaired behavioral tests than with either therapy alone. Furthermore, Cotten et al. [8] reported that transplantation of autologous UCB to neonates with HIE in addition to hypothermia was safe and feasible and showed better (74%) 1-year survival with Bayley scores >85 in UCB recipients compared with 41% in the concurrent cooled infants. Overall, these findings suggest that cell-based therapies combined with hypothermia might act synergistically and thus could be a novel therapy to improve prognosis of currently intractable neonatal severe HIE.

Protective Mechanisms of Stem Cell Transplantation for Bronchopulmonary Dysplasia

Engraftment and Differentiation

Due to their multilineage differentiation potential, the beneficial effects of stem cell transplantation have been initially ascribed to the transdifferentiation of donor cells into neuronal cells, thus reconstituting damaged brain parenchymal cells. However, this is a very rare in vivo event [7] and the numbers of engrafted donor cells observed are extremely low [17–19]. These findings suggest that direct donor cell engraftment, transdifferentiation and recruitment of damaged brain tissue with exogenously transplanted stem cells might not be the primary mechanism of neuroprotection.
Paracrine Effects

Both endothelial progenitor cells and conditioned media obtained from endothelial progenitor cells, but not vehicle media, showed significant potentiation of angiogenesis and neurogenesis after cerebral ischemia in mice [20]. More recently, exosomes derived from MSC, but not from fibroblasts, significantly attenuated the development of PHH, upregulated inflammatory responses, increased apoptotic cell death and astrogliosis, and reduced myelination (unpubl. data). Taken together these findings suggest that neuroprotective effects of stem cell transplantation might be predominantly mediated by paracrine rather than regenerative mechanisms. Use of the MSC secretome rather than stem cells shows excellent promise as a novel therapeutic approach for neonatal brain disorders as it could bypass the putative side effects, such as tumor formation, associated with live stem cell treatments.

Paracrine Mediators

Several paracrine factors secreted from MSC such as brain-derived neurotrophic factor (BDNF), nerve growth factor, vascular endothelial growth factor, insulin-like growth factor and interleukins have been known to enhance brain repair after hypoxia and/or ischemia [20–28]. However, the specific key paracrine factors secreted by transplanted stem cells that mediate the protective paracrine anti-inflammatory, antioxidative and anti-apoptotic activities in neonatal brain disorders have not been elucidated yet.

Combined DNA and antibody microarray analyses enable us to simultaneously interrogate the transcriptional and translational responses of MSC after severe IVH without any prior knowledge of the neuroprotective mechanism of MSC. Recently, we observed significant upregulation of BDNF both in DNA and antibody microarray analyses (unpubl. data). Moreover, knockdown of BDNF secreted by MSC via transfection with small interfering RNAs specific for human BDNF abolished the neuroprotective effects of MSC in severe IVH, such as significant attenuation of PHH, impaired behavioral test, increased apoptosis, inflammation and astrogliosis, and reduced myelination in the newborn rats. Therefore, these findings suggest that BDNF secreted from the donor cells might be one of the key paracrine factors that play pivotal roles in attenuating severe IVH-induced brain injuries in newborn rats.

Given the neuroprotective effects of BDNF secreted by the transplanted cells, transplantation of MSC overexpressing BDNF might be a more advantageous and promising therapeutic approach than naïve MSC transplantation alone. However, controversial therapeutic findings were observed with BDNF gene-modified MSC transplantation in ischemic brain lesions in rats [25, 29]. Another concern for clinical translation of BDNF gene-modified MSC transplantation is tumorigenicity. Although the risk of oncogenicity could be reduced by using adenovirus instead of lenti- or retrovirus, further studies will be necessary to investigate the therapeutic efficacy and also to attenuate the safety concerns relative to clinical use of gene-modified MSC.

Determining the Optimal Cell Type for Stem Cell Transplantation

Determining the most appropriate cell types and sources is critical for successful clinical translation of cell-based therapies into protection against neonatal brain injuries. Therapeutic efficacy of various stem cells including embryonic stem cells, inducible pluripotent stem cells, endothelial progenitor cells, neural stem cells and MSC for neonatal brain disorders have been extensively discussed in the literature [30–33]. However, it is very difficult to choose among the various types and sources of stem cells and to identify those that ultimately exhibit the best therapeutic efficacy in protecting against neonatal brain injuries.

UCB, a medical waste usually discarded at birth, is a rich source of mononuclear cells (MNC) that contains high levels of primitive multipotent stem/progenitor cells [34]. Improved neurobehavioral outcome with UCB transplantation was observed in the newborn rat model of HIE and in a phase I clinical study of neonatal HIE [8] which demonstrated that transplantation of autologous UCB obtained at birth was safe, feasible and potentially efficacious. These findings suggest that UCB MNC may be a good cell source for transplantation for neuroprotection against neonatal brain disorders. However, despite being easy to obtain, the quantity of UCB MNC obtained from each infant and the amount of stem cells within each batch of MNC are quite variable and heterogeneous. Therefore, it would be virtually impossible to standardize the optimal dose of UCB MNC. Overall, these findings suggest that UCB MNC might not be an ideal source as the ‘off-the-shelf’ drug for clinical application of cell-based therapies to treat neonatal brain disorders.

MSC are more ethically and socially acceptable than embryonic stem cells and they are immune privileged due to lack of MHC II antigens. Therefore, the therapeutic potential of MSC transplantation for neonatal brain disorders has been extensively investigated. Among various...
sources of MSC, donor age has been known to have a negative impact on the expansion and differentiation potential of MSC, and MSC derived from gestational tissues such as UCB, Wharton’s jelly or umbilical cord show lower immunogenicity [35, 36] and higher proliferative capacity [37, 38] and paracrine potency than adult tissue-derived MSC [39]. Moreover, human UCB-derived MSC produced in strict compliance with good manufacturing practices are available for clinical use [9]. These findings suggest that MSC derived from birth-associated tissues might be the optimal source for future clinical uses in protecting against neonatal brain disorders.

**Determining the Optimal Route for Stem Cell Transplantation**

Determining the optimal route for MSC transplantation is a key issue to be resolved for future successful clinical translation of stem cell therapies for protection against bronchopulmonary dysplasia. Currently, cell-based therapies have been given by local intraventricular [4–7], intrathecal [40], intranasal [41, 42], systemic intraperitoneal [43] or intravenous [5] administration. The systemic intravenous or intraperitoneal approach which is most convenient and minimally invasive shows distinct therapeutic advantages compared with the more invasive local intraventricular or intrathecal approach, especially in very unstable newborn infants with brain injuries who could not tolerate invasive local injection of stem cells. However, some animal data have suggested that the systemic route is not optimal for treating local brain lesions because transplanted cells might be retained in other organs such as lung, liver, spleen and kidney [44]. Systemically transplanted cells might also have limitations crossing the blood-brain barrier with resultant poorer stem cell delivery compared with local administration [5]. In our recent study [5], both delivery and therapeutic efficacy of local intraventricular transplantation of MSC in protecting against severe IVH were more than fivefold higher compared with systemic intravenous administration in newborn rats. Moreover, in the clinical setting, local transplantation of MSC is feasible via a ventricular tap without any further invasive operation because newborn infants have open anterior fontanelles. Overall, these findings suggest that local intraventricular or intrathecal rather than systemic intravenous or intraperitoneal transplantation of stem cells might be the more therapeutically effective delivery route for treating newborn infants with brain injuries.

**Determining the Optimal Dose for MSC Transplantation**

Determining the optimal dose for MSC transplantation is another important issue to be addressed for successful clinical translation. Donega et al. [42] demonstrated that intranasal transplantation of MSC dose-dependently attenuated hypoxic-ischemic brain injury in 9-day-old mice, showing best motor cognitive and histologic outcomes at a dosage of $1 \times 10^6$ cells, and identified $5 \times 10^5$ cells as the minimal effective dose. Recently, we have observed that the therapeutically effective dose for MSC transplantation could be reduced more than fivefold by choosing local intraventricular over systemic intravenous administration in protecting against severe IVH-induced brain injuries in newborn rats [5]. In light of these findings, further studies to determine the optimal dose of stem cell transplantation for their clinical benefit in newborn infants with neonatal brain disorders are anticipated.

**Determining the Optimal Timing for MSC Transplantation**

While recent study results suggest cell-based therapies as a novel therapy for neonatal brain disorders, determination of the optimal timing for stem cell transplantation is also another major issue that remains to be clarified for future clinical application. Beneficial effects of cell-based therapies have been noted with stem cell transplantation in the first hours to 7 days after brain injury [4–7, 45, 46]. In a neonatal mouse stroke model, intrastriatal injection of neural stem cells at 2 days but not at 7 days after injury significantly reduced ischemia-induced brain atrophy [47]. In a well-designed dose ranging and timing study in newborn mice, intranasal stem cell transplantation at 3 and 10 days, but not at 17 days, after hypoxic-ischemic brain injury improved outcomes [42]. In our recent study to optimize the timing of MSC transplantation for severe IVH in newborn rats, significant neuroprotection was demonstrated only with early (2 days) but not late (7 days) treatment after induction of severe IVH [48]. Overall, these findings suggest that the therapeutic time window for stem cell transplantation might be narrow and thus administration closer to the time of brain injury might provide better therapeutic outcomes. Further studies will be necessary to clarify this.
Long-Term Safety and Outcomes of Stem Cell Transplantation

Despite promising preclinical study results, a longitudinal study assessing long-term safety and therapeutic efficacy of stem cell transplantation in an animal model is essential for successful clinical translation. Long-term research using the rodent model is feasible in a short time frame due to its short life span. Intranasal transplantation of MSC in newborn mice was not associated with any neoplasia in the nasal turbinates, brain or other organs, and while the control group showed severe behavioral impairments, the treated animals had long-lasting improvements in histologic, sensorimotor and cognitive function at 14 months after hypoxia-ischemia [41]. Similarly, the protective and beneficial effects of intratracheal transplantation of MSC in hyperoxic lung injuries in newborn rats were sustained without any long-term adverse effects including tumor formation up to 70 days postnatally, which is comparable to human adolescence [41]. The number of MSC decreased drastically by 72 h after intranasal treatment [18] and less than 1% was detected at 18 days after intracranial administration [49], and virtually no MSC were detected at 70 days after intratracheal administration [17]. No long-term adverse effects including neoplasia of MSC transplantation might thus be attributable to the fact that they do not engraft in the brain and exert therapeutic function through a brief ‘hit and run’ mechanism following administration [50]. The long-lasting protective effects of MSC transplantation despite their fade away suggest that protection during the early critical time period of neonatal brain injury could result in long-term sustained neuroprotection. The data indicating sustained long-term protective effects of MSC transplantation without any long-term adverse effects warrant the translation of MSC transplantation into clinical studies for treatment of neonatal brain disorders.

Phase I Clinical Trial for Severe IVH

In previous preclinical translational studies to determine the therapeutic efficacy [4], optimal route [5] and timing [48] of human UCB-derived MSC transplantation in a neonatal newborn rat model of severe IVH, MSC were found to provide neuroprotection against severe IVH-induced neonatal brain lung injury. Based on the promising evidence from these preclinical studies in the experimental IVH model, a phase I dose-escalating clinical study on the safety and feasibility of human UCB-derived MSC transplantation in preterm infants with severe IVH has been designed and started (NCT02274428). This study is an open-label, single-center clinical trial to assess safety and feasibility of a single intraventricular transplantation of allogenic human UCB-derived MSC within 7 days after detection of severe (grade ≥3) IVH in preterm infants. The first 3 infants will be given a low dose (5 × 10^6 cells/kg in 1 ml/kg of saline) and the next 6 will be given a high dose (1 × 10^7 cells/kg in 2 ml/kg of saline) under ultrasound guidance. The primary outcome measures are unsuspected death or anaphylactic shock within 6 h after MSC transplantation and the secondary outcome measures are death or hydrocephalus requiring shunt surgery up to 1 year of age. We are also planning to conduct long-term follow-up studies of these enrolled infants. Favorable results from these current clinical trials and future phase II clinical trials are expected to pave the way for future clinical introduction of stem cell transplantation for currently intractable neonatal brain disorders such as severe IVH.

Conclusions

Various translational studies have broadened knowledge and understanding of stem cell therapy for neonatal brain injury, and clinical trials are harnessing the therapeutic potential of stem cell therapies for neonatal brain disorders. Exciting progress in both animal and clinical research has brought human stem cell therapy for neonatal brain disorders one step closer to clinical translation. However, a better understanding of the potential neuroprotective mechanisms of stem cells and the resolution of clinical issues such as clinical indication, optimal route, timing, dose and multiple modes of action are required to permit safe clinical translation of stem cell therapy for neonatal brain disorders.

Disclosure Statement

The authors have nothing to disclose.
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