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Research Paper

Preterm umbilical cord blood derived mesenchymal stem/stromal cells protect preterm white matter brain development against hypoxia-ischemia

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ABSTRACT

Introduction: Preterm infants are at high risk for white matter injury and subsequent neurodevelopmental impairments. Mesenchymal stem/stromal cells (MSC) have anti-inflammatory/immunomodulatory actions and are of interest for neural repair in adults and newborns. This study examined the neuroprotective effects of allogeneic MSC, derived from preterm umbilical cord blood (UCB), in a preterm sheep model of white matter injury.
Methods: Quad-lineage differentiation, clonogenicity and self-renewal ability of UCB-derived MSC were confirmed. Chronically instrumented fetal sheep (0.7 gestation) received either 25 min hypoxia-ischemia (HI) to induce preterm brain injury, or sham-HI. Ten million MSC, or saline, were administered iv to fetuses at 12 h after HI. Fetal brains were collected 10d after HI for histopathology and immunocytochemistry.
Results: HI induced white matter injury, as indicated by a reduction in CNPase-positive myelin fiber density. HI also induced microglial activation (Iba-1) in the periventricular white matter and internal capsule ($P < .05$ vs control). MSC administration following HI preserved myelination ($P < .05$), modified microglial activation, and promoted macrophage migration (CD163) and cell proliferation (Ki-67) within cerebral white matter ($P < .05$). Cerebral CXCL10 concentration was increased following MSC administration ($P < .05$), which was likely associated with macrophage migration and cell proliferation within the preterm brain. Additionally, MSC administration reduced systemic pro-inflammatory cytokine TNFα at 3d post-HI ($P < .05$).
Conclusions: UCB-derived MSC therapy preserved white matter brain structure following preterm HI, mediated by a suppression of microglial activation, promotion of macrophage migration and acceleration of self-repair within the preterm brain. UCB-derived MSC are neuroprotective, acting via peripheral and cerebral anti-inflammatory and immunomodulatory mechanisms.

1. Introduction

Infants born preterm have a high risk of brain injury, with surviving preterm infants commonly demonstrating white matter injury (WMI) (Volpe, 2003). WMI is characterized by sparse or disorganized axonal myelination, astrogliosis, and/or microglial activation, and can be detected in infants born preterm via magnetic resonance imaging. Although WMI has a complex etiology, it is well described that two relatively common upstream insults, hypoxia-ischemia (HI) and inflammation, are principal contributors towards brain injury (Volpe, 2008; Gopagondanahalli et al., 2016). WMI is known to underlie cerebral palsy, and 50% of infants with WMI will develop cognitive, behavioral and/or attention deficits (Volpe, 2003; Woodward et al., 2006). Due to the complex pathology and etiology of WMI, and the fragility of extremely preterm infants, there are currently no effective treatments available to prevent or repair WMI in preterm infants.

Mesenchymal stem/stromal cells (MSC) describe multipotent cells with anti-inflammatory/immunomodulatory properties, trophic influences on tissue repair and MSC are capable of differentiating into multiple lineages (Gang et al., 2004; Javazon et al., 2004; Trounson and McDonald, 2015). Due to their multi-potential mechanisms of action and relative ease of proliferation in vitro, human and nonhuman mammalian MSC have received attention as a therapeutic intervention for repair of neurological deficits (Trounson and McDonald, 2015;

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Barry and Murphy, 2004). Recent studies show that transplantation of MSC in both neonatal and adult animal models of brain damage promote functional and structural improvements (van Velthoven et al., 2012; van Velthoven et al., 2010a; Donega et al., 2013; Kim et al., 2012; Yasuhara et al., 2006; Lim et al., 2011; Lee et al., 2010). In neonatal rodent models of acute HI and stroke, MSC treatment improves behavioral outcomes, and reduces tissue loss via neuronal and oligodendrocyte regeneration, regardless of route of administration (van Velthoven et al., 2012; van Velthoven et al., 2010a; Donega et al., 2013; Kim et al., 2012). Early studies suggested that MSC might act therapeutically to replace damaged cells, differentiating into neurons and oligodendrocytes (Shen et al., 2007; Messerli et al., 2013); but current knowledge supports that MSC principally act to modify the microenvironment response to insult, thereby promoting endogenous repair processes (van Velthoven et al., 2009). In particular, recent studies demonstrate that MSC modify the microglia/macrophage transcriptional environment to assist in maintenance of homeostasis after traumatic brain injury, thereby reducing secondary injury, and promoting tissue repair and nerve regeneration (Zanier et al., 2014; Xu et al., 2017).

Multiple sources of MSC are available, including bone marrow, UCB, umbilical cord (UC), and adipose tissue (Kim et al., 2012; Xia et al., 2010). Compared to other sources of MSC, UCB-MSC are appealing due to their high proliferative capacity, low immunogenicity and strong immunosuppressive potential, with few ethical issues (Ruff et al., 2013; Deuse et al., 2011). A recent randomized, placebo-controlled trial investigating the safety and efficacy of human UCB-MSC infusion in children with cerebral palsy has shown promising results (Huang et al., 2018) and, currently, another randomized double-blind, placebo-controlled trial has been listed in the clinical trial database (NCT01988584). It is, however, generally accepted that UCB-MSC studies are complicated by low yield and great variability in cell number obtained from full-term UCB collection (Barachini et al., 2009; Kern et al., 2006; Manca et al., 2008). Interestingly, this may not be a factor in relation to preterm birth, with the proportion of MSC in preterm UCB significantly increased when compared to UCB collected from term birth (Javed et al., 2008; Jain et al., 2013; Erices et al., 2000) and, indeed, the number of MSC available for collection increases with decreasing preterm age at birth (Javed et al., 2008; Jain et al., 2013). Thus, collecting MSC from preterm UCB, rather than term UCB, may be more appropriate for clinical application and treatment of preterm WMI.

Accordingly, in this study we investigated the feasibility and efficacy for the administration of allogeneic, ex-vivo expanded MSC derived from preterm UCB for neuroprotective use in a well-established ovine fetal model of preterm WMI. Chronically instrumented preterm fetal sheep were exposed to 25 min of acute hypoxia ischemia (HI) induced via umbilical cord occlusion at 0.7 gestation, equivalent to brain development at 28–32 weeks in the human (Li et al., 2016). We set out to determine whether UCB-derived MSC were neuroprotective for preterm WMI, and to examine the mechanisms by which MSC mediate neuroprotective benefits in a preclinical large animal model of preterm WMI.

2. Materials and methods

2.1. Ovine UCB-MSC isolation and expansion

This study was approved by the Monash Medical Centre Animal Ethics Committee (MMCA/2013/17). Ovine UCB was collected from 5 normal preterm sheep undergoing cesarean-section at ~118d gestation. UCB samples were collected into 50 ml Falcon tubes containing heparin, and processed within 4 h of collection. After centrifuging the blood at 3100 rpm for 12 min at room temperature without brake, the buffy coat layer was isolated, and 20–30 million UCB cells were plated in a 100-mm dish (Falcon) at 37 °C in 5% CO_2 in a humid atmosphere under aseptic conditions in medium containing DMEM/F-12, 10% fetal bovine serum, 100 U/ml penicillin–streptomycin, 0.25μg/ml amphotericin B, and 2 mM L-glutamine (Life Technologies). At 24 h media was replaced, non-adherent cells discarded, and adherent cells retained. Medium was changed at 7d intervals thereafter. Between days 10–14, each colony consisting of spindle-shaped cells was harvested with 0.25% trypsin (Gibco) using cloning cylinders (Sigma-Aldrich), and transferred to individual wells in 24-well tissue culture plates (Costar 3524; passage 1). The cells were incubated with medium replaced every 3d, and harvested by trypsinization when they reached 80–90% confluence and re-plated (passage 2). From passage 2, the cells were incubated at seeding densities of 5000 cells/cm^2 in T75 or T175 culture flasks (Corning), and expanded. After passage 3, the cells were re-suspended in 10% fetal bovine serum and 10% DMSO (Merck), and cryopreserved in liquid nitrogen until required for administration.

2.2. Serial cloning assay

The clonogenicity and self-renewal ability of the MSC were examined by serial cloning in culture (Letouzey et al., 2015). Several of the largest individual clones on culture plates were collected by trypsinisation in cloning rings, and re-cloned. Cells were counted visually under a phase contrast microscope using an ocular grid, seeded at 10–20 cells/cm^2 onto the 10 cm dishes, and cultured in standard medium, changed every 7d, to generate secondary clones. Similarly, secondary, tertiary and quaternary clones were harvested and re-cloned as previously described (Letouzey et al., 2015). The cloning efficiency at each sub-cloning was assessed.

2.3. In vitro differentiation

The differentiation potential of the expanded ovine MSC (passage 3) into adipocytes, osteoblasts, chondrocytes and myocytes was evaluated. For adipogenic, osteogenic and myogenic differentiation, the cells were seeded separately at 5000 to 10,000 cells/cm^2 on coverslips (Thermo Scientific) in 24-well plates and cultured in specific differentiation medium, as described previously for human and ovine MSC (Letouzey et al., 2015). Briefly, to induce chondrogenic differentiation, 5×10^5 cells were cultured in 15 ml conical tubes following centrifugation and cultured in chondrogenic medium to produce a 3D micromass culture (Letouzey et al., 2015). Controls were ovine MSC cultured in standard medium. The cells were incubated for 4 weeks in differentiation or control media, replaced every 3d. Cells were then fixed on coverslips and stained with 4% Alizarin Red (pH 4.1), 1% Oil Red O or by immunohistochemistry using anti-α-smooth muscle actin antibody (3.6 μg/ml, clone 1A4; Dako) for the detection of calcification, lipid vacuoles deposition and α-smooth muscle actin expression to reveal osteogenic, adipogenic, and myogenic differentiation, respectively. Chondrogenic micromass cultures were fixed, processed and paraffin embedded. Sections were stained with 1% alcian blue (pH 2.5; Sigma-Aldrich) to detects acidic mucins(Letouzey et al., 2015). Stained cells or sections were examined using an Olympus BX41 microscope (Olympus), and images taken using an Olympus DP25 digital camera (Olympus).

2.4. Surface phenotype

Freshly expanded MSC at passage 3 were phenotypically analyzed. The cells were detached from the culture dish with trypsin, rinsed twice with phosphate buffered saline (PBS), incubated with fluorochrome-conjugated antibody in the dark at room temperature for 20 min, and rinsed twice with PBS. The following primary antibodies were used: FITC-conjugated CD44 (0.1 mg/ml, mouse IgG1; LSBio), FITC-conjugated CD45 (0.1 mg/ml, mouse IgG1; LSBio), AF647-conjugated CD73 (1 μg/ul, rabbit IgG; Bioss), PE-conjugated CD90 (1 μg/ul, rabbit IgG; Bioss), FITC-conjugated CD105 (1 μg/ul, rabbit IgG; Bioss), FITC-