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Research Report

Umbilical cord blood cells regulate the differentiation of endogenous neural stem cells in hypoxic ischemic neonatal rats via the hedgehog signaling pathway

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ABSTRACT

Transplantation of umbilical cord blood mononuclear cells (UCBMC) promotes the proliferation of endogenous neural stem cells (NSCs), but it has been unclear whether the proliferating NSCs can differentiate into mature neural cells. Therefore, we explored the effects of UCBMC transplantation on the differentiation of endogenous NSCs and their underlying mechanisms. Seven-day-old Sprague-Dawley rats underwent left carotid ligation followed by hypoxic stress. UCBMC were transplanted 24 h after hypoxia ischemia (HI). BrdU/β-tubulin/HNA/DAPI, BrdU/GFAP/HNA/DAPI, Ngn1/DAPI, and BMP4/DAPI were measured by immunofluorescence staining; Shh, Gli1, Ngn1, and BMP4 proteins were measured by western-blot analysis 28 days after transplantation. More newborn neurons and fewer astrocytes were observed in the HI+UCBMC group, its neuronal percentage was higher, and glial percentage was lower compared with the N+UCBMC ($P<0.05$) and HI+PBS groups ($P<0.01$), while fewer newborn neurons and more newborn astrocytes were found in the HI+cyclopamine (an antagonist of the hedgehog protein)+UCBMC group compared with the HI+UCBMC group ($P<0.01$). The expression of Shh, Gli1, and Ngn1 proteins was higher and BMP4 protein was lower in the HI+UCBMC compared with the HI+PBS group ($P<0.01$) and the HI+cyclopamine+UCBMC group ($P<0.01$). Linear regression analysis showed that the differentiation of NSCs correlated with expression of Ngn1 and BMP4 proteins ($P<0.01$). In conclusion, UCBMC promote neuronal differentiation and reduce glial differentiation in hypoxic ischemic neonatal rats via the hedgehog signaling pathway.

Abbreviations: BMP4, bone morphogenetic protein 4; BrdU, 5-bromo-2'-deoxyuridine labeling; DMSO, dimethyl sulphoxide; GFAP, glial fibrillary acidic protein; HI, hypoxia ischemia; HIE, hypoxic ischemic encephalopathy; HNA, human nucleus antigen; N, normal; Ngn1, neurogenin1; NSCs, neural stem cells; PBS, phosphate buffered saline; UCBMC, umbilical cord blood mononuclear cells

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1. Introduction

Hypoxic ischemic encephalopathy (HIE) is a common but serious disease among newborns during the perinatal period and can result in epilepsy, cerebral palsy, or retardation, creating heavy burdens for both the family and society (Mwaniki et al., 2012). There is still no effective treatment for HIE except the use of hypothermia as a neuroprotective strategy (Ma et al., 2012). It is well known that neural stem cells (NSCs) persist in certain locations in the brain throughout life and can be activated. Previous studies showed that hypoxia/ischemia (HI) can stimulate proliferation of endogenous NSCs in the subventricular zone and dentate gyrus areas, but brain damage still followed. This suggests that either the activated NSCs did not survive to become mature neurons (Brazel et al., 2005; Ikeda et al., 2005) or they differentiated into astrocytes (Horie et al., 2008). Therefore, it will be of great significance to determine how to induce the proliferating NSCs to differentiate into mature neurons. Other research has shown that under certain circumstances, the brain produces some inflammatory factors (Huehnchen et al., 2011), activating the expression of sonic hedgehog protein (the key protein in the Shh signaling pathway), which displays both morphogenic and mitogenic properties and can also repair tissue in different organs (Amankulor et al., 2009). The Shh signaling pathway has been closely associated with neurogenesis, and it will be of great significance if we learn how to activate it. Among umbilical cord blood mononuclear cells (UCBMC) are a large proportion of stem and progenitor cells (Carroll, 2012; Rosenkranz and Meier, 2011; Xiao et al., 2005) and UCBMC have been shown to secrete many cytokines, regulate the immune inflammation response (Hall et al., 2009), alleviate brain damage (Geissler et al., 2011; Xia et al., 2010), and promote the proliferation of NSCs (Gornicka-Pawlak el et al., 2011) via the hedgehog signaling pathway (Wang et al., 2013a, 2013b). However, it has remained unclear whether proliferating NSCs differentiate into mature neurons, or whether the differentiation mechanisms are associated with the hedgehog signaling pathway. If UCBMC transplantation promotes proliferating NSCs to differentiate into mature neurons, it would be of great importance to the treatment of HIE. Thus the possible mechanisms involved deserve further research.

Neurogenin1 (Ngn1), one of the members of the proneural basic Helix-Loop-Helix (bHLH family), is mainly expressed during cortical neurogenesis (Kim et al., 2008; Hirabayashi et al., 2009). Ngn1 has been shown to be an activator of neuronal transcription factor that promotes neurogenesis and inhibits glial differentiation (Lundell et al., 2009). The BMP family belongs to the TGF-β (transforming growth factor) superfamily and consists of more than 30 members (Bond et al. 2012), among which BMP4 has the potential to inhibit neurogenesis (Chalazonitis et al., 2011), stimulate the formation of astrocytes, and play an important role in regulating the differentiation of NSCs (Zhang et al., 2011). Therefore, in this study, we examine the effects of UCBMC transplantation on the differentiation of NSCs, measure the changes in expression of Ngn1 and BMP4, and explore the correlation between the differentiation of NSCs and Ngn1 and BMP4 proteins, offering a therapeutic basis for clinical application of UCMBC.

2. Results

2.1. Effects of UCBMC transplantation on the differentiation of NSCs in the cortex

The differentiation of NSCs was monitored using β-tubulin and GFAP as markers for mature neurons and astrocytes, respectively. As shown in Fig. 1, fewer $BrdU^+$β-$tubulin^+HNA^-DAPI^+$ cells were seen in the HI+PBS group than in the N+PBS group ($P<0.01$). More $BrdU^+$β-$tubulin^+HNA^-DAPI^+$ cells were seen in the HI+UCBMC group than in the HI+PBS or HI+cyclopamine+UCBMC groups ($P<0.01$). There were also more $BrdU^+$β-$tubulin^+HNA^-DAPI^+$ cells in the HI+UCBMC group than in either the N+UMBMC group ($P<0.01$) or the N+PBS group ($P<0.01$), but there was no significant difference between the HI+UCBMC and HI+DMSO+UCBMC groups ($P>0.05$).

More $BrdU^+GFAP^+HNA^-DAPI^+$ cells were found in the HI+PBS than in the N+PBS group ($P<0.01$). There were fewer $BrdU^+GFAP^+HNA^-DAPI^+$ cells in the HI+UCBMC group than in the HI+PBS and HI+cyclopamine+UCBMC group ($P<0.01$). There was still no significant difference in the number of $BrdU^+GFAP^+HNA^-DAPI^+$ cells between the N+UCBMC and HI+UCBMC groups ($P>0.05$), and there was also no significant difference in the number of $BrdU^+GFAP^+HNA^-DAPI^+$ cells between the HI+UCBMC and HI+DMSO+UCBMC groups ($P>0.05$).

The percentages of mature neurons and astrocytes were also calculated to compare the effects of UCBMC transplantation on the differentiation of NSCs. The percentage of mature neurons (i.e., $BrdU^+$β-$tubulin^+HNA^-DAPI^+$ cells) of the HI+PBS group (59.1±5.1%) was lower than in the N+PBS group (67.0±5.7%, $P<0.01$). The percentage of newborn mature neurons was higher in the HI+UCBMC group (79.39±6.8%) than in the N+UCBMC (72.5±5.6%, $P<0.05$), HI+PBS, or HI+cyclopamine+UCBMC groups ($P<0.01$). The percentage of newborn astrocytes (i.e., $BrdU^+GFAP^+HNA^-DAPI^+$ cells) (12.8±4.6%) was significantly lower in the cerebral cortex of the HI+UCBMC group than in the HI+PBS (37.4±8.9%), N+PBS (26.5±7.2%), or N+UCBMC (22.5±6.3%) groups ($P<0.01$). There was no significant difference in the percentage of either newborn mature neurons or astrocytes between the HI+UCBMC and HI+DMSO+UCBMC groups ($P>0.05$, $n=10$ in each group, Fig. 1).

2.2. Western blot analysis of Shh, Gli1, Ngn1, and BMP4 proteins

Western blots were analyzed for the Shh, Gli1, Ngn1, and BMP4 proteins in the lesioned brain homogenate of the four groups 28 days following transplantation. As shown in Fig. 2, western blots revealed five distinct bands, which may have corresponded to the Shh, Gli1, Ngn1, BMP4 and Glyceraldehyde-3-phosphate (GAPDH) antibodies. The HI+UCBMC group showed higher Shh and Gli1 protein expression than the N+PBS ($P<0.01$), HI+PBS ($P<0.01$), N+UCBMC ($P<0.01$), or HI+cyclopamine+UCBMC groups ($P<0.01$) in the injured hemisphere. There was no significant difference in the expression of Shh and Gli1 proteins