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Short communication

Changes in Interleukin-1 alpha serum levels after transplantation of umbilical cord blood cells in a model of perinatal hypoxic-ischemic brain damage

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SUMMARY

Transplantation of human umbilical cord blood (hUCB) cells is a potential approach for the treatment of perinatal hypoxic-ischemic brain injury. Neurological and motor deficits resulting from the brain lesion are ameliorated upon transplantation. The molecular mechanisms underlying these improvements are currently being unravelled. One parameter identified as part of the beneficial effects of hUCB cells is the reduction of brain inflammation. It is, however, unclear whether the modulation of brain inflammation is due to local or systemic effects of hUCB cells.

In this study, the effects of hUCB cell transplantation in a model of perinatal hypoxic-ischemic brain injury were investigated at the systemic level by measurement of serum levels of pro-inflammatory cytokines by multiplex bead arrays.

Two days after induction of the brain damage, levels of the pro-inflammatory cytokines Interleukin-1α (IL-1α), Interleukin-1β (IL-1β), and Tumor necrosis factor α (TNFα) were increased in the serum of rats. Application of hUCB cells, in turn, correlated with a reduced elevation of serum levels of these pro-inflammatory cytokines. This decrease was accompanied by a reduced expression of CD68, a marker protein of activated microglia/macrophages in the brain.

Therefore, systemic modulation of the immune response by hUCB cells could represent one possible mechanism of how these cells might mediate their beneficial effects. Creation of a regenerative environment with reduced inflammation might account for the functional regeneration observed upon hUCB cell treatment in lesioned animals.

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1. Introduction

Perinatal hypoxia-ischemia severely affects children and is an important cause for mortality und morbidity in this early period of life. Depending on the extent and location of the insult, mental impairment, ataxia, epilepsy, permanent neurological and motor deficits are the consequences (Volpe, 2008, 2009). As efficient clinical or pharmaceutical strategies to prevent or to reduce the incidence and the impact of hypoxic-ischemic brain injury in the perinatal period are limited, cell therapy is one promising approach for treatment (Bennet et al., 2012).

The therapeutic potential of human umbilical cord blood (hUCB) cell transplantation in ischemic diseases of the nervous system has been demonstrated in many experimental animal models (Rosenkranz and Meier, 2011), including one of perinatal hypoxic-ischemic brain injury (Meier et al., 2006; Pimentel-Coelho et al., 2010; Rosenkranz et al., 2012; Yasuhara et al., 2010). Transplantation of hUCB cells led to amelioration of lesion impaired neurological and motor functions, as assessed by walking pattern analysis and determination of neurological scores (Geissler et al., 2011; Meier et al., 2006; Wasielewski et al., 2012; Yasuhara et al., 2010). In some studies, these beneficial effects of cord blood cell transplantation were associated with at least the transient presence of transplanted cells at the lesion site (Geissler et al., 2011; Meier et al., 2006; Rosenkranz et al., 2010), whereas in other studies, injured brains were devoid of human cells (Borlongan et al., 2004; Yasuhara et al., 2010). Thus, indirect effects might be responsible for the beneficial action of hUCB cells.

As inflammation seems to contribute to the fatal effects of brain ischemia in general (Amantea et al., 2009; Arvin et al., 1996; Streit et al., 1999) and perinatal hypoxia-ischemia in particular (Meier et al., 2006; Northington et al., 2011), it might pose one target for the action of hUCB cells. In fact, effects of hUCB cells on the number of active microglia cells in the damaged brain have been described

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(Pimentel-Coelho et al., 2010; Wasielewski et al., 2012). In addition, hUCB cells were shown to secrete a large number of cytokines in culture (Neuhoff et al., 2007; Newman et al., 2006) and among these are a number of cytokines with known anti-inflammatory potential (Bai et al., 1997; Li et al., 2005; Nikkhah et al., 1993). Thus, the release of anti-inflammatory cytokines by hUCB cells, if also sustained upon transplantation, might influence the extent of inflammation after hypoxic-ischemic brain injury. As long term (weeks to months) presence of hUCB cells at the lesion site in animal models of perinatal hypoxia-ischemia is no prerequisite for their action, one might consider systemic effects of these cells via cytokine secretion.

This study therefore focuses on the investigation of potential systemic effects of transplanted hUCB cells using a model of hypoxic-ischemic brain injury in newborn rats. The serum levels of pro-inflammatory cytokines were investigated in a rat model of hypoxic-ischemic brain injury and compared between animals with and without transplantation of hUCB cells at two time points.

Our data show that hUCB cells have a systemic influence on inflammation in that they reduce levels of the pro-inflammatory cytokines Interleukin-1α (IL-1α), Interleukin-1β (IL-1β), and Tumor necrosis factor α (TNFα) in the serum. In the brain, the increase in CD68 expression was also reduced upon hUCB cell transplantation. Both the reduced concentration of pro-inflammatory cytokines and the reduced expression of CD68, indicating fewer activated microglial cells/macrophages in the brain, might be due to the secretion of anti-inflammatory cytokines by intraperitoneal transplanted hUCB cells in vivo. The results of this study indicate that hUCB cells potentially modulate the immune system in general and that these effects, in turn, may support functional regeneration of the lesioned brain.

2. Materials and methods

2.1. Preparation of hUCB-derived mononuclear cells for transplantation

Blood from the umbilical cord and placenta was obtained from the Department of Gynecology and Obstetrics (St. Elisabeth-Hospital, Bochum, Germany) after receiving the mother's written informed consent. The umbilical vein was punctured post partum, and the blood was collected in umbilical cord blood collection bags containing citrate phosphate dextrose as anticoagulant (Maco Pharma, Langen, Germany) and stored at 18 °C up to 48 h until further processing. Preparation of the mononuclear cell fraction was performed by Ficoll Paque (GE Healthcare, Munich, Germany) density gradient centrifugation according to the manufacturer's instructions. The mononuclear fraction of cells was collected from the interphase, and resuspended in phosphate buffered saline (PBS). Viability of mononuclear cells was 97 ± 2% as determined by toluidine blue staining. In the manuscript text, human umbilical cord blood-derived mononuclear cells are referred to as "cord blood cells" or "hUCB cells".

The local Ethics Committee of the Ruhr-University Bochum approved use of cord blood samples.

2.2. Animals and hypoxic-ischemic injury surgical procedure

All surgical and experimental protocols were approved by the appropriate institutional review committee (LANUV Recklinghausen, Germany) and met the guidelines of the German animal protection law.

The Levine model (Levine, 1960; Rice et al., 1981) was used to achieve reproducible hypoxic-ischemic injury in neonatal Wistar rats on postnatal day (P) seven and was performed as described previously (Meier et al., 2006). Briefly, seven-day-old Wistar rat pups were deeply anesthetized by inhalation of isoflurane. The left common carotid artery was exposed, double ligated, and severed. To induce systemic hypoxia, the pups were exposed to a hypoxic gas mixture (8% oxygen/92% nitrogen) for 80 min. The environmental temperature was strictly maintained at 36 °C. Sham animals were anesthetized and the left common carotid artery was exposed, but not ligated. Sham animals did not undergo systemic hypoxia.

Transplantation of hUCB-derived mononuclear cells (1×10^7/500 μl 0.9% NaCl), or vehicle (NaCl) was performed by intraperitoneal injection 24 h after the insult as established in previous studies (Chen et al., 2001; Meier et al., 2006) without any further use of anaesthesia or immunosuppressant.

At P9 or P21, rats were anaesthetized and decapitated. Brains were dissected and macroscopic brain injury was assessed immediately (Bona et al., 1997). All further studies and quantification were performed in a blinded fashion.

Experimental animal groups were sham = no lesion; sham + hUCB = no lesion following transplantation of hUCB cells; lesion = animals with hypoxic-ischemic brain injury following transplantation of 500 μl 0.9% NaCl/H_2O (v/v) (vehicle); lesion + hUCB = animals with hypoxic-ischemic brain injury following transplantation of hUCB cells.

2.3. Histological and Immunohistochemical analysis

Brains were covered in tissue freezing medium (Leica, Nussloch, Germany) and cryopreserved. Histology and immunohistochemistry were performed on cryosections of 12 μm thickness. Precise localization of the lesion areas was assured by histological staining according to Kluever-Barrera, showing myelinated fiber tracts in light green and cell bodies in blue. Immunohistochemistry was performed as described previously (Rosenkranz et al., 2010) using anti-CD68 (ED1; 1:100; AbD Serotec, Düsseldorf, Germany) as primary and Alexa Fluor™ 488 conjugated goat-anti-mouse (1:3000; Life Technologies, Darmstadt, Germany) as secondary antibody. Fluorescence was documented using conventional fluorescence microscopy (Zeiss 200 M inverted microscope). Data were exported as TIFF files into Adobe Photoshop CS2 (Adobe Imaging Systems, USA).

2.4. Immunoblots

Immunoblot analysis was performed as described previously (Neuhoff et al., 2007). Proteins (10 μg/lane) were separated on 10% SDS-polyacrylamid gels. After electrophoretic transfer of proteins, nitrocellulose membranes were incubated with primary antibodies anti-CD68 (ED1; 1:100; AbD Serotec) or anti-β-Actin (1:10,000; Sigma–Aldrich, Taufkirchen, Germany) at 4 °C overnight. After washing, immunoblots were incubated with horseradish–peroxidase-conjugated secondary antibodies (1:8000; Jackson ImmunoResearch, Newmarket, UK) for 1 h. Visualization was performed by enhanced chemiluminescence detection (GE Healthcare, München, Germany).

Quantification was carried out by densitometric analysis of signal intensities (Quantity One, Bio-Rad Laboratories, München, Germany) using β-Actin expression for normalization.

2.5. Rat cytokine non-magnetic bead assay

Blood samples were taken from the four experimental groups at both time points (P9 and P21). The samples were separated using BD Microcontainer™ (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instructions. The sera were collected and stored at −70 °C until further processing. To measure cytokine levels in blood serum of the four different experimental

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